DOI 10.1002/mnfr.200600202 Mol. Nutr. Food Res. 2007, *51*, 324–332

Research Article

Inhibition of gastric H^+,K^+-ATPase and *Helicobacter pylori* growth by phenolic antioxidants of *Zingiber officinale*

Mugur N. Siddaraju and Shylaja M. Dharmesh

Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore, Karnataka, India

Ulcer is a common global problem characterized by acute gastric irritability, bleeding, *etc.* due to either increased gastric cell proton potassium ATPase activity (PPA) or perturbation of mucosal defence. *Helicobacter pylori* has been identified as a major ulcerogen in addition to oxidative stress and nonsteroidal anti-inflammatory drugs. In this paper, we report ginger-free phenolic (GRFP) and ginger hydrolysed phenolic (GRHP) fractions of ginger (*Zingiber officinale*) as potent inhibitors of PPA and *H. pylori* growth. GRFP and GRHP inhibited PPA at an IC_{50} of 2.9 ± 0.18 and 1.5 ± 0.12 μg/mL, exhibiting six- to eight-fold better potency over lansoprazole. GRFP is constituted by syringic (38%), gallic (18%) and cinnamic (14%) acids and GRHP by cinnamic (48%), *p*-coumaric (34%) and caffeic (6%) acids as major phenolic acids. GRFP and GRHP further exhibited free radical scavenging (IC_{50} 1.7 ± 0.07 and 2.5 ± 0.16), inhibition of lipid peroxidation (IC_{50} 3.6 ± 0.21 and 5.2 ± 0.46), DNA protection (80% at 4 μg) and reducing power abilities (80–338 U/g) indicating strong antioxidative properties. GRFP and GRHP may thus be potential in-expensive multistep blockers against ulcer.

Keywords: Antioxidant activity / Free and bound phenolics / *Helicobacter pylori* / H^+,K^+-ATPase / *Zingiber officinale*

Received: October 12, 2006; revised: November 30, 2006; accepted: December 2, 2006

1 Introduction

Dietary plant phenolic compounds have been described to exert varieties of biological actions such as free radical (FR) scavenging, metal chelation, modulation of enzymatic activity, *etc*. [1–3] and they receive particular attention due to their putative role in the prevention of several human diseases. Phenolic acids and their derivatives are widely spread in plants [4] and a number of phenolic acids are linked to various cell wall components such as arabinoxylans, proteins [5, 6] and pectic polysaccharides [7]. Free and bound phenolics are known to play a crucial role in the defence mechanism, offering protection against oxidative stress (OS) caused by both biotic and abiotic factors. Phenolics from grape seed [8], cacao liquor [9] and prickly pear [10] have been shown to possess antiulcer activity. The antioxidant activity of phenolics may be an important contributing antiulcer factor since free radicals (FR)/reactive oxygen species (ROS) are related to the occurrence of ulcers [11].

Ginger (*Zingiber officinale*) belonging to the family Zingiberaceae is cultivated in various parts of the world, especially in India, China and Mexico. It is used since time immemorial as a dietary component in daily life as a food spice and Ayurvedic; Chinese and traditional medicine systems have recorded ginger as an important medicinal plant. Ginger has been reported to exert antioxidant and antiulcer [12, 13], antiemetic [14], anti-inflammatory, antitumour [15], carminative, diaphrodic, digestive, expectorant, as well as gastro protective [16] activities. The powdered rhizome contains 3–6% fatty oil, 8–9% protein, 60–70% carbohydrates, 3–8% crude fibre, about 7–8% ash, 9–12% water and 2–3% volatile oil [17, 18]. However, only volatile oils such as linalool, zingiberol, zingiberene and oleore-

Correspondence: Dr. Shylaja M. Dharmesh, Department of Biochemistry and Nutrition, CFTRI, Mysore 570 020, Karnataka, India
E-mail: cancerbiolab@yahoo.co.in
Fax: +91-0821-2517233

Abbreviations: DPPH, 1,1-diphenyl-2-picryl hydrazyl; **FR,** free radicals; **GAE,** gallic acid equivalent; **GRFP,** ginger-free phenolics; **GRHP,** ginger hydrolysed phenolics; **MIC,** minimal inhibitory concentration; **OS,** oxidative stress; **PC,** parietal cells; **PPA,** proton potassium ATPase activity; **PPI,** proton potassium ATPase inhibition; **TBA,** 2-thiobarbituric acid; **TBARS,** thiobarbituric acid reactive substances

sins-gingerol, shogaol have been reported to possess antioxidant activity [19, 20] and very little information is available on the phenolics of ginger and their potential contribution to antiulcer activity. It is possible that some phenolics may be included in volatile oils and in turn may contribute to the total antioxidant activity also.

Ulcer-gastric hyperacidity is a common problem causing sufferings to human kind. Hyperacidity is a pathological condition due to uncontrolled hypersecretion of hydrochloric acid from the parietal cells (PC) of gastric mucosa through the proton pumping by H^+,K^+-ATPase (PPA) harboured on the plasma membrane of PC [21]. The imbalance between mucosal defensive factors such as gastric mucin and acidity results in gastric ulcer. For hypersecretion status such as Zollinger-Ellission syndrome where there is a high and uncontrolled production of acid, the use of nonsteroidal anti-inflammatory drugs (NSAID), infection by *Helicobacter pylori via* damaged mucin layer, and repeated OS conditions are the main causative factors.

Reactive oxygen species such as superoxide anion, hydrogen peroxide and hydroxyl radicals generally implicated in tissue damage [22] also appears to be responsible for gastric ulcer pathogenicity [23] and a number of clinical experimental data suggested that gastritis and gastric ulcer by alcohol [24], NSAID [25] and by *H. pylori* [26] are mediated through ROS. In the current study, we addressed the problem of isolation of antioxidant fraction – free and bound phenolics from ginger, an economical source compared to the earlier reported sources (grapes, cocoa liquor and prickly pear) and evaluated their potential antiulcer effect by examining proton potassium ATPase blockade and anti-*H. pylori* properties.

2 Materials and methods

2.1 Plant material

Ginger (*Z. officinale*) rhizome was purchased from the local market at Mysore, India and used for studies. Fresh ginger rhizome (1 kg) was cleaned, washed under running tap water, cut into small pieces, air dried, powdered for particle size of 20 mesh, isolated for free and bound phenolics and proton potassium ATPase inhibition (PPI), antioxidant and anti-*H. pylori* activity were determined. Since bound phenolics were released during sample preparation, the term hydrolysed is used for phenolics in the bound fraction for appropriate understanding. Henceforth, bound phenolics will be referred to as hydrolysed phenolics.

2.2 Chemicals

Agarose, calf thymus DNA, phenolic acid standards such as gallic, tannic, caffeic, *p*-coumaric, ferulic, gentisic, protocatechuic, syringic and vanillic acids, synthetic antioxidants such as butylatedhydroxyanisole (BHA) and butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA), and 1,1-diphenyl-2-picryl hydrazyl (DPPH) were obtained from Sigma (St. Louis, MO). Folin-Ciocalteu reagent, ferric chloride, trichloroacetic acid, sodium carbonate, ferrous sulphate and ascorbic acid were of the highest quality purchased from Qualigens Fine Chemicals (Mumbai, India). HPLC column (Shimpak C_{18}) was obtained from Shimadzu (Kyoto, Japan) and HPLC grade solvents employed for HPLC analysis were purchased from Spectrochem Biochemicals (Mumbai, India).

2.3 Isolation of free and hydrolysed phenolic fractions of ginger

Free phenolics were extracted according to the method of Ayumi *et al*. [27]. Briefly, 2 g of ginger powder was extracted with 4×50 mL each of 70% ethanol and centrifuged at $3000 \times g$ for 10 min at room temperature. The clear supernatant was concentrated by flash evaporation (Buchi 011, Switzerland), 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 sulphate to remove the moisture, filtered and evaporated to dryness and taken in 2 mL of methanol w/v, designated as ginger-free phenolic fraction (GRFP). The experiment was performed in triplicate to evaluate the yield with statistical significance.

Hydrolysed phenolics were extracted according to the method of Nordkvist *et al*. [28]. Two grams of 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 $3000 \times g$ 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 phenolic acid and it was designated as ginger hydrolysed phenolic fraction (GRHP). Extraction was performed in triplicate to evaluate the yield with statistical significance.

2.4 Estimation of total phenolic content

The Folin-Ciocalteu reagent assay was used to determine the total phenolic content [29]. 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, mixed in a cyclo mixer. The absorbance was measured at 765 nm with a Shimadzu UV-Visible spectrophotometer (Shimadzu UV-160 spectrophotometer) after incubation for 2 h at room temperature. Gallic acid was used as

a standard for the calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligram *per* gram sample. The assay was performed in triplicate.

2.5 HPLC analysis of phenolic antioxidants of GRFP and GRHP

Phenolic acids of GRFP and GRHP were analysed by HPLC (model LC-10A, Shimadzu) on an RP Shimpak C_{18} column (4.6 mm × 250 mm, Shimadzu) 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 [30]. Phenolic acid standards such as caffeic, coumaric, cinnamic, ferulic, gallic, gentisic, protocatechuic, syringic and vanillic acids were employed for identification of phenolic acids present in GRFP and GRHP by comparing the retention time under similar experimental conditions.

2.6 Determination of H^+,K^+-ATPase inhibition (PPI) by GRFP and GRHP fractions in comparison with lansoprazole (PPA inhibitor)

Fresh sheep stomach was obtained from local slaughterhouse at Mysore, India. The mucosa of gastric fundus was cut off and the inner layer was scraped for parietal cells (PC) [31], 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 ginger phenolics, GRFP and GRHP in a reaction mixture containing 16 mM Tris buffer (pH 6.5) and the reaction was initiated by adding to the 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 inorganic phosphate (Pi) released/hour at various doses (2 – 10 μg) of GRFP and GRHP. Results were compared with known antiulcer proton potassium ATPase inhibitor drug lansoprazole and with standard phenolic acids, since the active fraction of ginger contained phenolic acids.

2.7 Anti-*H. pylori* activity

H. pylori was obtained from endoscopic samples of ulcer patients from KCDC (Karnataka Cardio Diagnostic Centre, Mysore, India) and cultured on Ham's F-12 nutrient agar medium with 5% FBS at 37°C for 2 – 3 days in a microaerophelic condition [32]. *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 also confirmed by the growth of culture in the presence of susceptible and resistant antibiotics.

2.7.1 Agar diffusion assay

H. pylori activity was tested by the standard agar diffusion method [33]. Briefly, the Petri plates were prepared with Ham's F-12 nutrient agar media containing 5% FBS inoculated with 100 μL of *H. pylori* culture (10^5 cells/mL). Sterile discs of high-grade cellulose of diameter 5.5 mm were impregnated with 20 μL of known extract at 5, 10 and 15 μg/disc of GRFP and GRHP placed on the inoculated Petri plates. Amoxicillin was used as positive reference standard and 0.9% saline as negative control. For comparative evaluation, discs containing 10 μg each of amoxicillin, GRFP and GRHP were used in addition to the control. *H. pylori* growth inhibition was determined as the diameter of the inhibition zones around the discs. The growth inhibition diameter was an average of four measurements taken at four different directions and all the tests were performed in triplicates.

2.7.2 Minimal inhibitory concentration (MIC)

MIC values were determined by conventional broth dilution method [33]. Serial dilutions (final volume of 1 mL) of GRFP and GRHP (0.05 – 50 μg/mL) were performed with 0.9% saline. Following this, 9 mL of Ham's F-12 nutrient medium with 5% FBS was added. Broths were inoculated with 100 μL of *H. pylori* suspension (5×10^4 CFU) and incubated for 24 h at 37°C. Amoxicillin was used as a positive control since *H. pylori* is susceptible to amoxicillin and 0.9% saline as negative control. After 24 h, *H. pylori* growth was assayed by measuring absorbance at 625 nm. MIC was defined as the lowest concentration in micrograms of GAE to restrict the growth to $A < 0.05$ at 625 nm (no macroscopic visible growth).

2.8 Measurement of antioxidant activity in GRFP and GRHP

2.8.1 FR scavenging activity

The antioxidant activity of GRFP and GRHP, on the basis of the scavenging activity of the stable DPPH-FR, was determined by the method described by Braca *et al.* [34]. An aliquot of 100 μL of GRFP and GRHP at various concentrations – 1 – 5 μg/mL were 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:

$$\text{Scavenging effect}(\%) = \left(\frac{\text{A 517 nm (control)} - \text{A 517 nm (sample)}}{\text{A 517 (control)}}\right) \times 100$$

2.8.2 Reducing power ability

The reducing powers of GRFP and GRHP were determined according to the method of Yen and Chen [35]. The extract of GRFP and GRHP (1 – 5 μg/mL) were 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 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 indicated increased reducing power.

2.8.3 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). A 10% fresh liver homogenate was prepared in 20 mM PBS, pH 7.4 [36]. Briefly, 0.25 mL of liver homogenate was incubated with 2 – 10 μg/mL of GRFP and GRHP in 20 mM PBS, pH 7.4. After 5 min of pretreatment, 0.5 mL each of ferric chloride (400 mM) and 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, 0.37% TBA in 0.25 N HCl) and tubes were boiled for 15 min at 95°C, cooled, centrifuged and read at 532 nm. TBARS was measured by using a standard TMP (1,1,3,3-tetramethoxy propane) calibration curve (0.1 – 0.5 μg) and expressed as percent inhibition of lipid peroxidation by extracts.

2.8.4 DNA protection assay

The DNA protective effect of phenolic fractions was determined electrophoretically (Submarine Electrophoresis System, Bangalore Genei, Bangalore, India) using calf thymus DNA [37]. Calf thymus DNA (1 μg in 15 μL) was subjected to oxidation by Fentons 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, Germany) and the intensity of the bands was determined (Easywin software). Protection to DNA was calculated based on the DNA band corresponding to that of the native in the presence and absence of 2 and 4 μg of GRFP and GRHP.

2.9 Statistical analysis

Generally, the analyses were repeated three times and all the data were expressed as mean value ± SD. The comparisons between mean values were tested using Duncan's new multiple range test at a level of $p \leq 0.05$ [38].

3 Results and discussion

The phenolic acids and their antiulcer, antioxidant activities in ginger-free and -bound phenolic fractions warranted a thorough investigation. Generally, phenolics have the tendency to bind to various biomolecules particularly to polysaccharides and hence are possible to exist in bound form in natural resources. Varieties of dietary sources studied previously in our laboratory indicated the presence of various health beneficial phenolic compounds [30, 39]. In this study, we report the potential ulcer preventive ability of free and bound phenolics of ginger by evaluating antioxidant, anti-*H. pylori*, proton pump inhibition and DNA protective ability.

3.1 Total phenol content

The total phenolic content in GRFP and GRHP phenolic fractions was found to be 2.6 ± 0.24 mg GAE/g and 1.1 ± 0.07 mg GAE/g of dried, defatted ginger powder, respectively. Approximately 3 – 5% yield of phenolics was noticed in ginger phenolic fractions (Table 1).

Table 1. Total phenolic content and yield of phenolics *per* 100 g (w) of GRFP and GRHP fractions

Phenolic fraction	Yield (mg/g) dry wt.	Percent yield (g/100 g)	Phenolic content (mg/g)
GRFP	$3.2^{b} \pm 0.30$	0.32^{b}	$2.6^{b} \pm 0.24$
GRHP	$1.8^{a} \pm 0.08$	0.18^{a}	$1.1^{a} \pm 0.07$

Free and bound phenolic constituents were isolated from 2 g of defatted dry powder as *per* the protocol mentioned under Section 2. A 2 mg powder was solubilized in methanol and phenol was estimated in soluble fraction; and to calculate the yield from 2 g of ginger powder, total solubles were dried and weighed. All data are the mean ± SD of three replicates, mean value followed by different letters (a, b) in the same column are significantly different ($p = 0.05$).

3.2 Identification of phenolic acids in GRFP and GRHP

HPLC analysis indicated phenolic acid composition in both GRFP and GRHP fractions of ginger (Fig. 1). The major phenolic acids present in GRFP were syringic acid (38%) followed by gallic acid (18%) and cinnamic acid (14%). Small amounts of caffeic (10%), gentisic (6%), protocatechuic (4%) and ferulic (4%) acids were also found to be present. GRHP fractions contained cinnamic acid (48%) and *p*-coumaric acid (34%) as major phenolic acids. Caffeic

(6%), syringic (4%), and gallic (2%), gentisic (2%) and protocatechuic (1%) acids were present in small amounts (Table 2). It is worth emphasizing here that cinnamic acid is one of the potential phenolic acids in inhibiting gastric H^+,K^+-ATPase enzyme as well as *H. pylori* growth.

3.3 Inhibition of gastric H^+,K^+-ATPase activity by GRFP and GRHP

H^+,K^+-ATPase is a regulatory enzyme found in the plasma membrane of PCs involved in passage of protons into the lumen of stomach causing acidity leading to ulcers. Ginger phenolic fractions: GRFP and GRHP inhibited gastric H^+,K^+-ATPase activity in a concentration-dependent manner (Fig. 2). Concentration required to inhibit 50% of H^+,K^+-ATPase activity is designated as IC_{50} and GRFP and GRHP showed an IC_{50} of 2.9 ± 0.18 and 1.5 ± 0.12 μg/mL, respectively; when compared to IC_{50} of 19.3 ± 2.2 μg/mL of lansoprazole (Table 2), a known proton pump inhibitor. Thus, phenolic fractions were found to be good inhibitors of the enzyme and the inhibition could be due to the binding

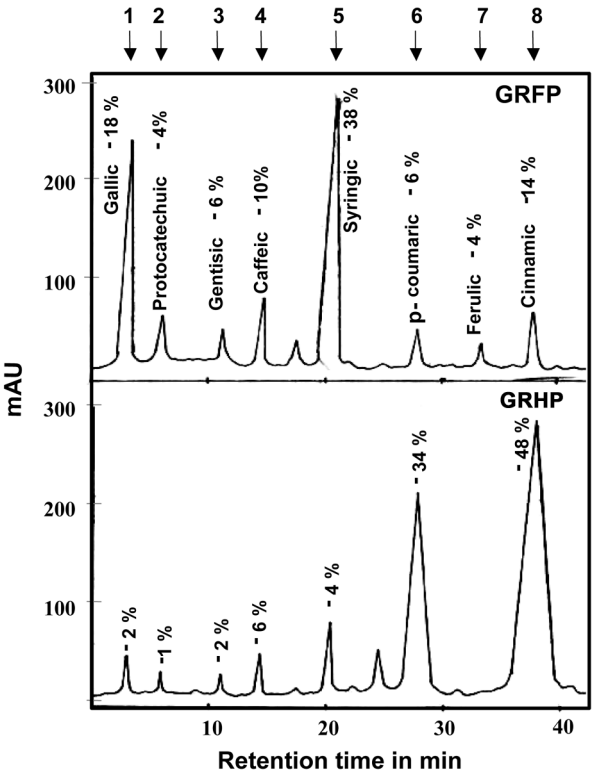


Figure 1. HPLC analysis of phenolic acid constituents in free (GRFP) and hydrolysed (GRHP) phenolic fractions. A 1 mg/mL solution of GRFP/GRHP was prepared, after vortexing for 10 min at room temperature with the mobile phase-water/acetic acid/methanol 80:5:15 v/v/v, isocratic and 20 μL of each was loaded on to HPLC Shimpak C_{18} column (4.6 mm × 250 mm, Shimadzu). A 20 μL of mg/mL standard phenolic acids was loaded independently and their specific retention time (min) was established. Phenolic acids in each fraction were identified comparing with their retention time with known standards. Arrows as 1–8 indicate the location as retention time in min standard phenolic acids.

Table 2. H^+,K^+-ATPase inhibition and antioxidant activity in GRFP, GRHP; comparison with standard phenolic acids and known antiulcer drug

Sample/standard phenolic acids	PPA activity IC_{50} (μg)	AOX potency* IC_{50} (μg)
Cinnamic acid	$15.1^{b} \pm 1.80$	$4.60^{bc} \pm 0.34$
Caffeic acid	$27.1^{cde} \pm 3.10$	$1.80^{ab} \pm 0.14$
Ferulic acid	$33.6^{de} \pm 3.80$	$6.60^{c} \pm 0.51$
Syringic acid	$37.4^{de} \pm 4.10$	$64.90^{d} \pm 5.42$
p-Coumaric acid	$39.7^{e} \pm 3.20$	$1.90^{ab} \pm 0.20$
Protocatechuic acid	$47.1^{f} \pm 4.20$	$1.35^{a} \pm 0.16$
Gentisic acid	$59.1^{g} \pm 6.10$	$3.00^{b} \pm 0.28$
Gallic acid	$132.0^{h} \pm 14.0$	$1.10^{a} \pm 0.09$
GRFP	$2.9^{a} \pm 0.18$	$1.70^{ab} \pm 0.07$
GRHP	$1.5^{a} \pm 0.12$	$2.50^{b} \pm 0.16$
Lansoprazole	$19.3^{bc} \pm 2.20$	–

GRFP and GRHP containing different phenolic acids at 2–10 μg range of phenolics were examined for PPI and AOX. In order to understand the probable contribution of identified phenolic acids in ginger phenolic fractions, under similar experimental conditions PPI and FRS activity was also performed for standard pure phenolic acids. Values are expressed as IC_{50} in μg/mL.

* AOX potency determined by FR scavenging activity. All data are the mean ± SD of three replicates, mean value followed by different letters (a, b, c, d, e, f, g, h) in the same column are significantly different ($p \leq 0.05$).

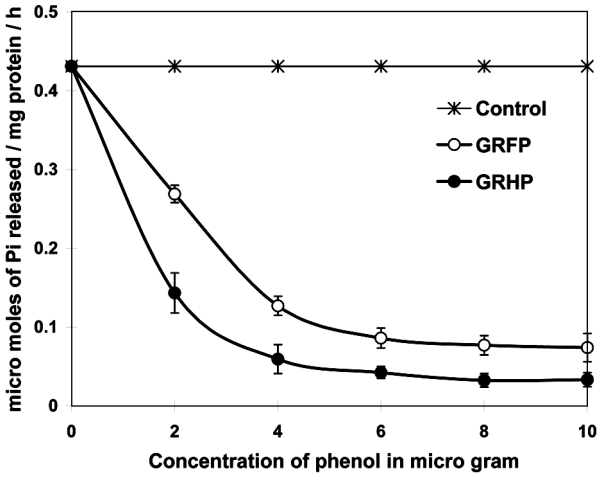


Figure 2. Inhibition of proton potassium ATPase enzyme activity by free (GRFP) and hydrolysed (GRHP) phenolic fractions of ginger. Sheep PC extract was employed as gastric-H^+,K^+-ATPase source, and activity was determined employing the protocol described under Section 2. A 350 μg enzyme protein/mL of reaction volume was incubated with 2–10 μg GAE of GRFP (○) and GRHP (●) and with no inhibitor (*). Enzyme activity is represented as micromoles of Pi released/mg enzyme protein/h. All the data are the mean ± SD of triplicate results.

of phenolic acids to ATPase enzyme similar to those of other phenolics [8]. In order to understand the probable ability of individual phenolic acids present in extracts in inhibiting gastric H^+,K^+-ATPase, pure phenolic acids were examined for their ability to inhibit the enzyme. As indicated in Table 2, 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} 47.1 μg/mL), gentisic (IC_{50} 59.1 μg/mL) and gallic acid (IC_{50} 132.1 μg/mL).

The fact that GRHP inhibited H^+,K^+-ATPase better (IC_{50} of 1.5 ± 0.12 μg/mL) than GRFP (IC_{50} of 2.9 ± 0.18 μg/mL), corroborates with the presence of increased levels of cinnamic acid in GRHP (48 μg/g) than in GRFP (14 μg/g). Higher content of *p*-coumaric acid (34 μg/g) in GRHP may also contribute significantly towards H^+,K^+-ATPase inhibition. *p*-Coumaric acid indeed is also a potent H^+,K^+-ATPase inhibitor with an IC_{50} of 39.7 μg/mL, although ~ two-fold less potent than lansoprazole. It is indicated both in the literature and from our study that H^+,K^+-ATPase is upregulated in ulcer condition [40], inhibition of the same therefore would result in gastric protection or antiulcer property. In the current study, the ability of GRFP and GRHP in inhibiting H^+,K^+-ATPase has therefore been revealed and implicated in their antiulcer potentials.

3.4 Inhibition of *H. pylori*

H. pylori obtained is a Gram-negative, acid tolerant, microaerophelic bacterium that lives in the stomach and duodenum [41]. *H. pylori* has coevolved with human and this species is indigenous to stomach of more than 5% population [42]. The most chronic infection of *H. pylori* is asymptomatic and if the colonization of bacteria persists, symptoms appear in 15–20% of the infected population and are associated with gastric ulcers. The bacteria isolated from endoscopic samples were Gram-negative, motile and showed positive for urease, catalase and oxidase tests (Table 3) [43]. Furthermore, it was confirmed by the response to antibiotics as it was resistant to antibiotics like erythromycin, nalidixic acid, polymixin B, penicillin and vancomycin; and was susceptible to amoxicillin, clarithriomycin and metronidazole. The appearance of a characteristic white mucilaginous colony confirms the identity of the bacteria as *H. pylori*. In the current study, we have investigated the ability of phenolic fractions of ginger to inhibit the growth of *H. pylori*. The initial antibacterial activity against *H. pylori* was assayed by agar diffusion method. Both the phenolic fractions showed anti-*H. pylori* activity. GRFP and GRHP showed a clear inhibition zone around the disc at 10 μg/mL concentration (Figs. 3A.4 and A.3), equivalent to that of a susceptible antibiotic – amoxicillin at 10 μg/mL (Fig. 3A.2). Figure 3A.1 depicts the control where uniform growth of *H. pylori* was observed. To quantitate the inhibitory effect of *H. pylori*, the diameter of growth inhibition area was measured and expressed in millimetres and is represented in Fig. 3B.

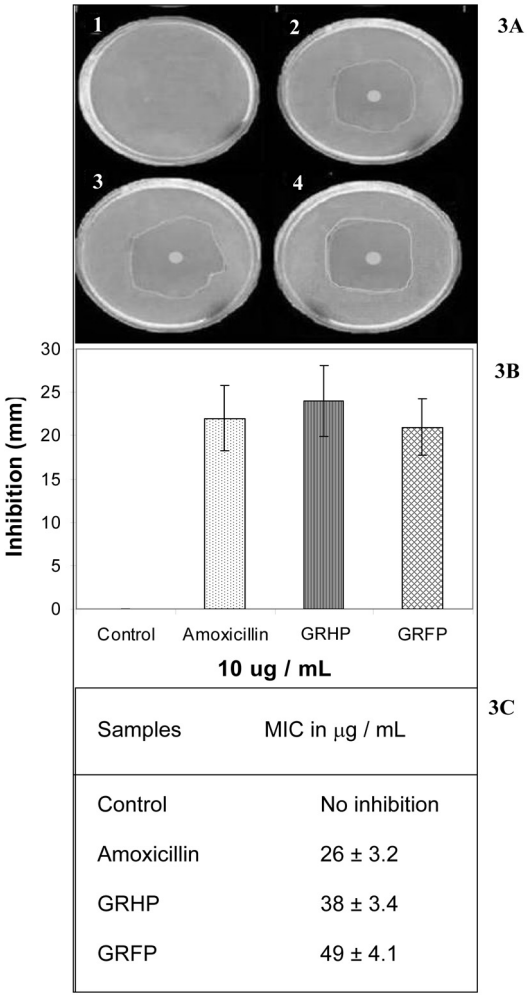
Table 3. Characteristic biochemical tests for *H. pylori*

Tests	Results
Urease	+ve
Catalase	+ve
Oxidase	+ve
Gram staining	Gram-negative
Motility	Motile
Colony characteristic	White mucilage type
Antibiotics	
Erythromycin	Resistant
Nalidixic acid	Resistant
Polymixin	Resistant
Penicillin	Resistant
Vancomycin	Resistant
Amoxicillin	Susceptible
Clarithromycin	Susceptible
Metronidazole	Susceptible

H. pylori obtained from endoscopic excision was subjected to various biochemical tests to confirm the identity of isolated bacteria as *H. pylori.* Gram staining, enzyme analysis and morphological analysis as well as antibiotic resistance/susceptibility were included in the tests for the characterization of *H. pylori.*

In view of the result obtained by the disc diffusion method, the MIC values were also determined by broth dilution method. The MIC values obtained confirm the significant ($p = 0.003$) anti-*H. pylori* activity, with MIC values – GRFP 49 ± 4.1 μg/mL and hydrolysed phenolics 38 ± 3.4 μg/mL (Fig. 3C). Results are further supported by an observation of Tabak [44] and Vattem *et al*. [45], where phenolic phytochemicals such as cinnamic acid, cinnamaldehyde, coumarins and flavonoids have been suggested to exhibit high anti-*H. pylori* activity. GRHP with higher content of cinnamic and coumaric acid showed better inhibition than GRFP. Phenolics were thought to exert their antimicrobial effect by causing (i) hyperacidification at the plasma membrane interface of the microorganism [46] or (ii) intracellular acidification, resulting in disruption of H^+,K^+-ATPase required for ATP synthesis of microbes [47] or (iii) may be related to inactivation of cellular enzymes causing membrane permeability changes [48]. The rate of inactivation of microbial cellular enzymes is dependent on the rate of penetration of phenolic antioxidants into the cell. In case of *H. pylori*, 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 [49].

Ginger has been known to contain gingerols and other bioactive compounds such as zingiberene, zingiberol, *etc*. Gingerol appears to be a major constituent (1–3%) contributing to antiulcer, anti-*H. pylori* [50] and antioxidant [51] activities. In the current study, however, phenolics extrac-



3C

Samples	MIC in μg / mL
Control	No inhibition
Amoxicillin	26 ± 3.2
GRHP	38 ± 3.4
GRFP	49 ± 4.1

Figure 3. Effect of GRFP and GRHP on *H. pylori* growth. (A) Anti-*H. pylori* activity was tested by the standard agar diffusion method. Discs (5.5 mm) containing 10 μg each of Amoxicillin – a known antibiotic (3A.2), GRHP (3A.3) and GRFP (3A.4) were impregnated with agar and 3A.1 served as control with no inhibitor disc. Clear area around the disc represents the inhibition zone due to the effect of the test fraction; (B) comparative effect of ginger phenolic fractions on the inhibition of *H. pylori* growth; (C) minimum inhibitory concentration as established by serial dilution technique. All the data are the mean ± S D of triplicate fractions.

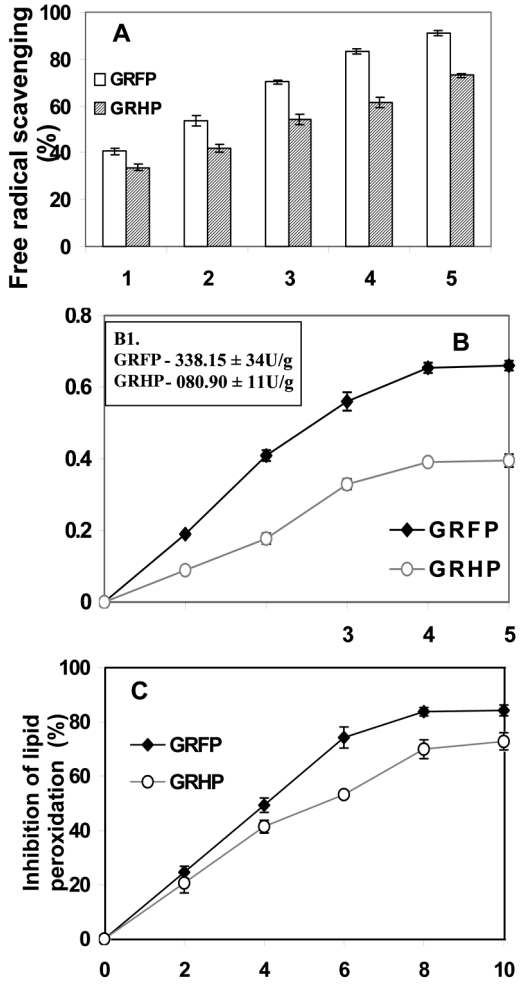


Figure 4. Antioxidant potency of GRFP and GRHP. Concentration of 1–5 and 2–10 μg GAE/mL of GRFP and GRHP were examined for FR scavenging (A) and reducing power (B). Total reducing power/g of sample is expressed as B1 in the inset of (B) and inhibition of lipid peroxidation (C) as *per* the protocol described under Section 2. All the data are the mean ± S D of triplicates.

tion excludes such reported bioactive compounds since they are volatile and hydrophobic in nature. Current study thus may emphasize the role of phenolics as they offer ~1.6-fold better contribution to antiulcer/anti-*H. pylori*/antioxidant activity besides their stability under extraction/physiological conditions.

3.5 GRFP and GRHP exhibited multipotent antioxidant activity

In order to provide an evidence that phenolic fractions also possess antioxidant activity, which is essential to counteract oxidative stress (OS)-induced ulcers, antioxidant potencies were determined.

3.5.1 FR scavenging activity

The scavenging activity of GRFP and GRHP on DPPH-FR was determined which primarily evaluates the proton radical scavenging ability of the phenolic compounds. DPPH is a stable, FR compound that possesses a proton-FR with a characteristic absorption at 517 nm which decreases significantly on exposure to proton radical scavengers [52]. It is well established that DPPH-FR scavenging by antioxidants is mainly due to their hydrogen-donating ability [53]. In the present study, GRFP and GRHP showed concentration-dependent radical scavenging activity (Fig. 4A). GRFP showed better radical scavenging activity with IC_{50} of 1.7 ±

0.07 μg/mL compared to GRHP – 2.5 ± 0.16 μg/mL. The scavenging activity was directly attributed to their phenolic content, since we have shown antioxidant potencies of pure phenolic acids, which are the representative antioxidant components of ginger extracts.

3.5.2 Reducing power assay

The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant property by breaking the FR chain by donating hydrogen atom [54]. The reducing power of the sample increased with an increase in concentration of phenolics (Fig. 4B). Approximately four-fold increase in the total reducing power was observed in GRFP than GRHP with 338.15 ± 34 and 80.9 ± 11 U/g, respectively. Thus, the data presented here may indicate that the phenolic fractions tested may act as reductones by donating electrons to FRs and thereby convert FRs to a more stable product and terminates FR chain reaction.

3.5.3 Inhibition of lipid peroxidation

Lipid peroxidation generates a number of degradation products, such as malondialdehyde (MDA), hexanol, *etc.* and is found to be an important cause of cell membrane destruction and cell damage [55]. MDA is a highly reactive species and crosslinks DNA with protein and thus damages the cells [56], disrupts its activity leading to chronic diseases. In the present study, we measured the potential of GRFP, GRHP to inhibit lipid peroxidation products (TBARS). The hydroxy radical generated through the Fenton reaction was scavenged by coincubation of rat liver homogenate with varying concentration (2 – 10 μg GAE/g sample) of GRFP and GRHP (Fig. 4C). GRFP showed maximum inhibition of lipid peroxidation with an IC_{50} 3.6 ± 0.21 μg GAE/g sample compared to GRHP (5.2 ± 0.46 μg GAE/g sample).

3.5.4 DNA protection activity

The Fenton's reagent causing DNA fragmentation (as visualized by an increased electrophoretic mobility of DNA) was recovered with the treatment of GRFP and GRHP extracts prior to OS. A dose-dependent protection was observed by both free and bound phenolics of ginger at 2 – 4 μg GAE, respectively (Fig. 5). A significant (>80%, $p \leq 0.005$) protection to native DNA during oxidation in the presence of these fractions was observed. These results indicate that free and bound phenolics of ginger can quench FRs and thereby may protect the DNA against OS-induced damage.

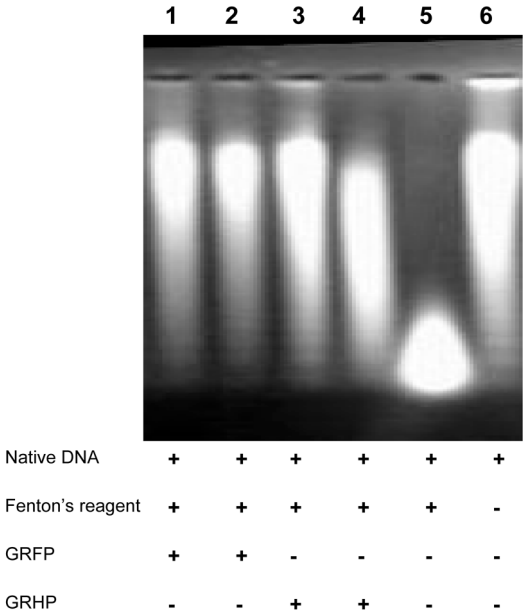


Figure 5. DNA protection ability of free (GRFP) and bound (GRHP) phenolics of ginger: 1 μg of native calf thymus DNA in (lane 6); 1 μg of DNA treated with Fenton's reagent (lane 5); DNA pretreated with 2 – 4 μg of GRFP (lanes 1 and 2) and GRHP followed by Fenton's reagent (Lanes 3 and 4) were loaded on to the 1% agarose gel. Bands were visualized by staining with ethidium bromide; and in the transilluminator, increased mobility represents DNA damage.

4 Concluding remarks

Ginger (*Z. officinale*) contains a significant amount of phenolics 2.6 ± 0.24 mg GAE/g and 1.1 ± 0.07 mg GAE/g as both free and hydrolysed forms, respectively. Both free and hydrolysed phenolic fractions of ginger were found to be effective in inhibiting H^+,K^+-ATPase activity, *H. pylori* growth and oxidative routes, which are responsible for the prevention of ulcer. Further, cinnamic, syringic and *p*-coumaric acids present in phenolic fractions of ginger may contribute significantly to H^+,K^+-ATPase as well as *H. pylori* inhibition, while other major phenolic acids like gallic acid found in a free phenolic fraction of ginger together with other phenolic acids may contribute to antioxidant activity (Table 1). Thus, both free and hydrolysed phenolics of ginger may find better application in ulcer management and ulcer therapy.

The authors thank Dr. V. Prakash, Director, Central Food Technological Research Institute, for his keen interest in the work and encouragement. Authors are thankful to Karnataka Cardiac Diagnostic Centre, Mysore, for providing H. pylori. Siddaraju thanks Council of Scientific and Industrial Research, India, for Junior Research Fellow/Senior Research Fellowship.

5 References

[1] Editorial, *Nutr. Rev.* 1994, *52*, 59 – 61.

[2] Koshihara, Y., Neichi, T., Murota, S., Lao, A., *et al.*, *Biochim. Biophys. Acta* 1984, *792*, 92 – 97.

[3] Reddy, S., Aggarwal, B. B., *FEBS Lett*. 1994, *341*, 19 – 22.

[4] Deshpande, S. S., Sathe, S. K., Salunkhe, D. K., Chemistry and safety of plant polyphenols, in: Friedman, M. (Ed.), *Nutritional and Toxicological Aspects of Food Safety*, Plenum, New York 1984, pp. 457 – 495.

[5] Harris, P. J., Hartley, R. D., *Nature* 1976, *259*, 508 – 510.

[6] Hartely, R. D., Morrison, W. H., Himmelsbach, D. S., Borneman, N. S., *Phytochemistry* 1990, *29*, 3701 – 3709.

[7] Bunzel, M., Ralph, J., Steinhart, H., *Czech. J. Food Sci*. 2004, *22*, 64 – 67.

[8] Saito, M., Hosoyama, H., Ariga, T., Katapka, S., Yamaji, N., *J. Agric. Food Chem*. 1998, *46*, 4113 – 4117.

[9] Galati, E. M., Mondello, M. R., Giuffrida, D., Dugo, G., *et al*., *J. Agric. Food Chem.* 2003, *51*, 4903 – 4908.

[10] Osakabe, N., Sanbongi, C., Yamagishi, M., Takizawa, T., Osawa, T., *Biosci. Biotechnol. Biochem*. 1998, *62*, 1535 – 1538.

[11] Das, D., Bandyopadhyay, D., Bhattacharjee, M., Banerjee, R. K., *Free Radic. Biol. Med.* 1997, *23*, 8 – 18.

[12] Hirahara, F., *Jpn. J. Nutr.* 1974, *32*, 1 – 39.

[13] Lee, L. K., Ahn, S. Y., *Korean J. Food Sci. Technol.* 1985, *17*, 55 – 57.

[14] Sharma, S. S., Kochupillai, V., Gupta, S. K., Seth, S. D., Gupta, Y. K., *J. Ethnopharmacol.* 1997, *57*, 93 – 96.

[15] Katiyar, S. K., Agrwal, R., Mukhtar, H., *Cancer Res.* 1996, *56*, 1023 – 1030.

[16] Al-Yahya, M. A., Rafatullah, S., Mossa, J. S., Ageel, A. M., *et al*., *Am. J. Chin. Med.* 1989, *17*, 51 – 56.

[17] Govindarajan, V. S., *Crit. Rev. Food Sci. Nutr.* 1982, *17*, 1 – 96.

[18] Mustafa, T., Srivastava, K. C., Jensen, K. B., *J. Drug Dev.* 1993, *6*, 25 – 39.

[19] Masuda, Y., Kikuzaki, H., Hisamoto, M., Nakatani, N., *Biofactors* 2004, *21*, 293 – 296.

[20] Yamahara, J., Mochizuki, M., Rong, H. Q., Matsuda, H., Fujimura., H., *J. Ethnopharmacol.* 1998, *23*, 299 – 304.

[21] Phull, P. S., Green, C. J., Jacyna, M. R., *Am. J. Gastroenterol. Hepatol.* 1995, *7*, 265 – 274.

[22] Halliwell, B., Hulliridge, J. M., *Methods Enzymol.* 1990, *186*, 1 – 85.

[23] Perry, M. A., Wadhwa, S., Parks, D. A., Pickard, W., Granger, D. N., *Gastroenterology* 1986, *90*, 362 – 367.

[24] Pihan, G., Regillo, C., Szabo, S., *Dig. Dis. Sci*. 1987, *32*, 1395 – 1401.

[25] Hawkey, G. M., Cole, A. T., Mc Intyre, A. S., Long, R. H., Hawkey, C. J., *Gut* 2001, *49*, 372 – 379.

[26] Warren, J. R., Marshall, B. J., *Lancet* 1983, *1*, 1273 – 1275.

[27] Ayumi, H., Masatsune, M., Seiichi, H., *Food Sci. Technol. Res.* 1999, *4*, 74 – 79.

[28] Nordkvist, E., Salomonsson, A. C., Aman, P., *J. Sci. Food Agric.* 1984, *35*, 657 – 661.

[29] Singleton, V. L., Rossi, J. A., *Am. J. Enol. Vitic*. 1965, *16*, 144 – 158.

[30] Suresh Kumar, G., Harish Nayaka, M. A., Shylaja, M. D., Salimath, P. V., *J. Food Comp. Anal*. 2006, *196*, 446 – 452.

[31] Sachs, G., Chang, H. H., Robon, E., Schackman, R., *et al*., *J. Biol. Chem.* 1976, *251*, 7690 – 7698.

[32] Traci, L. T., David, J. M. G., Harry, L. T. M., *J. Clin. Microbiol.* 2001, *39*, 3842 – 3850.

[33] Iris, C. Z., Marta, A. V., Maria, I. L., *J. Ethnopharmacol*. 2005, *102*, 450 – 456.

[34] Braca, A., Fico, G., Morelli, I., De Simone, F., *et al*. *J. Ethnopharmacol.* 2003, *86*, 63 – 67.

[35] Yen, G. C., Chen, H. Y., *J. Agric. Food Chem.* 1995, *43*, 27 – 32.

[36] Ohkawa, M., Ohishi, N., Kunio, Y., *Anal. Biochem.* 1979, *95*, 351 – 358.

[37] Rodriguez, H., Akman, S. A., *Electrophoresis* 1998, *5*, 646 – 652.

[38] Steel, R. G. P., Torrie, J., H., *Principles and Proceeding of Statistics*, Mc Graw, New York 1980.

[39] Leela, S., Shalini, V. K., Shylaja, M., *Arch. Biochem. Biophys.* 1992, *292*, 617 – 623.

[40] Sachs, G., Shin, J. M., Briving, C., Wallmark, B., Hersey, S., *Annu. Rev. Pharmacol. Toxicol.* 1995, *35*, 277 – 305.

[41] Marshall, B. J., Warren, J. R., *Lancet* 1984, *1*, 1311 – 1315.

[42] Mitchel, H., Mégraud, F., *Helicobacter* 2002, *7*, 8 – 16.

[43] Parsonnet, J., *Rev. Infect. Dis. Clin. North Am.* 1998, *12*, 185 – 197.

[44] Tabak, L. A., *Ann. Rev. Physiol.* 2005, *57*, 547 – 564.

[45] Vattem, D. A., Lin, Y. T., Ghaedian, R., Shetty, K., *Process Biochem*. 2005, *40*, 1583 – 1592.

[46] Shetty, K., Labbe, R. L., *Asia Pac. J. Clin. Nutr.* 1998, *7*, 270 – 276.

[47] Vattem, D. A., Lin, Y. T., Labbe, R. G., Shetty, K., *Innovative Food Sci. Emerg. Technol.* 2004, *5*, 81 – 91.

[48] Shahidi, F., Naczk, M. (Eds.), Nutricional and pharmacological effects of food phenolics, *Phenolics in Food and Nutraceuticals*, CRC Press LLC, New York 2004, pp. 331 – 402.

[49] Catherine, C., Mc Gowan, Timothy, L. C., Martin, J. B., *Gastroenterology* 1990, *110*, 926 – 938.

[50] Mahady, G. B., Pendland, S. L., Yun, G. S., Lu, Z. Z., Stoia, A., *Anticancer Res.* 2003, *23*, 3699 – 3702.

[51] Hirahara, F., *Jpn. J. Nutr.* 1974, *32*, 1 – 39.

[52] Yamaguchi, T., Takamura, H., Matoba, T., Terao, J., *Biosci. Biotech. Biochem.* 1998, *62*, 1201 – 1204.

[53] Chen, C. W., Ho, C. T., *J. Food Lipids 1995*, *2*, 35 – 46.

[54] Siddhuraju, P., Mohan, P. S., Becker, K., *Food Chem*. 2002, *79*, 61 – 67.

[55] Yoshikawa, T., Naito, Y., Tanigawa, T., Yoneta, T., *et al*., *Free Radic. Res. Comm*. 1991, *14*, 289 – 296.

[56] Kubow, S., *Trends Food Sci. Technol.* 1990, *1*, 67 – 72.

