Curcuma longa Extract Protects against Gastric Ulcers by Blocking H_2 Histamine Receptors

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Curcuma longa **has been commonly used as a traditional remedy for a variety of symptoms such as inflammation, gastritis and gastric ulcer. When *C. longa* extract was administered *per os* to pylori-ligated rat stomachs, it reduced gastric acid secretion and protected against the formation of gastric mucosal lesions. We therefore tested whether *C. longa* extract inhibits gastric ulcers by blocking the H_2 histamine receptor. Dimaprit, a H_2 histamine receptor agonist, induced intracellular cAMP production in U937 and HL-60 promyelocytes. Pretreatment with *C. longa* extract significantly blocked dimaprit-induced cAMP production in a concentration dependent manner, but had no effect on the elevation of cAMP levels triggered by isoproterenol-induced β_2-adrenoceptor activation in U937 cells. To identify the active component(s) of *C. longa* extract, we sequentially fractionated it by extraction with ethyl acetate, *n*-butanol and water. We found that the ethyl acetate extract showed the most potent H_2R antagonistic effect against dimaprit-induced cAMP production. However, curcumin, a major component of *C. longa* extract, showed no H_2R blocking effect. *C. longa* ethanol extract and ethylacetate extract also blocked the binding of [^{3}H]-tiotidine to membrane receptors on HL-60 cells. These findings suggest that the extract from *C. longa* specifically inhibits gastric acid secretion by blocking H_2 histamine receptors in a competitive manner.**

Key words *Curcuma longa*; H_2 histamine receptor; gastric ulcer; gastric juice secretion

Curcuma longa (*C. longa*), a common Indian dietary pigment and spice, has been used therapeutically in traditional folk medicine for a wide range of ailments, including wound healing, urinary tract infection, and liver ailments.[1,2] Although evidence suggests that a *C. longa* extract also has considerable gastroprotective and antiulcerogenic effects,[3,4] its mechanism of action remains unclear.

The genes encoding four histamine receptor subtypes (H_1, H_2, H_3 and H_4) have been cloned, and the receptors have been pharmacologically characterized, along with their signaling pathways.[5] The H_2 histamine receptor (H_2R) is a seven transmembrane G protein-coupled receptor (GPCR) coupled to the enzyme adenylyl cyclase, which produces the intracellular second messenger, cyclic AMP.[6] H_2R, which is expressed by various tissues, including the brain, stomach, heart, and lung, is involved in histamine-induced gastric acid secretion.[7] In gastric parietal cells especially, H_2R triggers the secretion of acid (HCl) into the stomach lumen through cAMP/protein kinase A/proton pump pathway.[8] Hence, H_2R blockers may be useful for treating diseases that involve gastric acid hyper-secretion, such as stomach ulcers and reflux esophagitis.

We hypothesized that the gastroprotective and antiulcerogenic effects of *C. longa* extract may be related with the H_2R signaling pathway. We found that this extract selectively inhibited the activation of H_2R and protected the stomach from gastric acid induced ulcers.

MATERIALS AND METHODS

Materials Dimaprit was purchased from Tocris (Bristol, U.K.). Histamine, ranitidine, Ro20-1724 were obtained from Sigma (St. Louis, MO, U.S.A.). [^{3}H]-cAMP were obtained from NEN Life Science Products (Boston, MA, U.S.A.). Tiotidine was obtained from Tocris Cookson Inc. (Ballwin, MO, U.S.A.). [3H]-Tiotidine were obtained from Perkin Elmer Life Sci. (Wellesley, MA, U.S.A.). RPMI-1640, penicillin–streptomycin and fetal calf serum were purchased from JBI (Daegu, Korea). cAMP-binding protein (CBP) were obtained from NEURONEX (Pohang, Korea). Pure curcumin was kindly provided by Dr. M. J. Kim in POSTECH.

Preparation of the *C. longa* Extract The ground dried *C. longa* (100 g) was fluxed with 80% ethanol (EtOH) (400 ml) and shaken at room temperature for 24 h. The extraction was performed twice. After extraction, the mixture was concentrated with rotary vacuum evaporator (EYELA, Japan). The EtOH extract was dissolved in H_2O (100 ml) and fractionated with organic solvents, ethyl acetate (EA) and *n*-butanol (*n*-Buta). Each dried material resuspended in DMSO for cellular experiment and in saline for *in vivo* test.

Animals Male Sprague–Dawley (SD) rats were kept, under controlled temperature (23 °C) and lighting (12 h : 12 h light : dark) conditions, with free access to water and food.

Cell Culture The U937 and HL-60 cell line (American Type Culture Collection, Rockville, MD, U.S.A.) was cultured in suspension at 37 °C in RPMI 1640 medium supplemented with 10% (v/v) of heat-inactivated fetal calf serum and 1% (v/v) penicillin–streptomycin in a humidified atmosphere of 95% air and 5% CO_2.

Measurement of [^{3}H]cAMP Intracellular cAMP generation was determined by using [^{3}H]cAMP competition assay kit (Neuronex, Korea). U937 and HL-60 cells were stimulated with dimaprit or isoproterenol for 20 min in the presence of the phosphodiesterase inhibitor Ro20-1724 (5 μM),

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and the reaction was quickly terminated by three repeated cycles of freezing and thawing. The samples were then centrifuged at 2500×***g*** for 5 min at 4 °C. The cAMP assay is based on the competition between [^{3}H]-labeled cAMP and unlabeled cAMP present in the sample for binding to a crude cAMP binding protein. Free [^{3}H]cAMP was adsorbed onto charcoal and removed by centrifugation. Bound [^{3}H]cAMP in the supernatant was then determined by liquid scintillation counting. Each sample was incubated with 50 μl [^{3}H]-labeled cAMP (5 μCi) and 100 μl binding protein for 2 h at 4 °C. Separation of protein-bound cAMP from unbound cAMP was achieved by adsorption of free cAMP onto charcoal (100 μl) followed by centrifugation at 12000×***g*** at 4 °C. The 200 μl supernatant was then placed into an Eppendorf tube containing 1.2 ml scintillation cocktail to measure radioactivity. The cAMP concentration in the sample was determined based on a standard curve and expressed as picomoles per number of cells.

[^{3}H]-Tiotidine Binding The binding of [^{3}H]-tiotidine to intact HL-60 cells was quantified by the method described in previous report[9] with some modification. Triplicate assays were performed in polyethylene tubes in 50 mM Tris–HCl, pH 7.4. [^{3}H]-Tiotidine were incubated with 10^6 cells/tube in the absence or presence of ranitidine or *C. longa* extracts in a total volume of 200 μl. After 40 min at 4 °C, incubation was stopped by dilution with 3 ml of ice-cold 50 mM Tris–HCl, pH 7.4; rapid filtration onto Whatman GF/B glass-fibers filters was performed under reduced pressure, followed by three washes with 3 ml of ice-cold buffer. The amount of bound radioactivity was measured in a liquid scintillation cocktail. Specific binding was defined as the difference in the amount of radioactivity bound in the absence and presence of 1 mM unlabeled tiotidine.

Measurement of Gastric Acid Secretion and Gastroprotective Activity *in Vivo* The pylorus-ligated rat model first described by Shay *et al.* was used with some modification.[10] SD rats weighing 200—250 g were used. Rats were deprived of food, but not water, for 18—24 h prior to each experiment. The test substances (vehicle, ranitidine, *C. longa* extract) dissolved in saline were administered orally (*per os*). After 1 h, rats were anesthetized by light ether, a small abdominal incision was made, the pylorus was ligated. The animals were sacrificed 8 h after ligation of the pylorus, the stomach was clamped at the oesophageal and duodenal junctions and then rapidly removed. The gastric juice was collected and the excised stomach was then filled with 15 ml of 4% formalin. After 24 h, the fixed stomach was opened along the greater curvature, gently rinsed in saline, and then pinned open to expose the gastric mucosa. The haemorrhagic and ulcerative lesions were counted and measured with a light microscope by an observer who was blinded to the treatment. The ulcer index was then calculated as the sum of the length of all lesions as previously described.[11] The ulcer index was determined in six animals in each animal group. The gastric content was placed in tubes for later centrifugation at 1500×***g*** for 30 min in a refrigerated centrifuge. Total acidity was determined by simple titration with 0.01 N NaOH using phenol-red as acid-base indicator.

Histological Examination The histological study of the stomach was performed following evaluation of the ulcer index. Samples of the corpus were excised and transferred to fresh formalin and later processed by routine techniques prior to embedding in paraffin. Sections (5 μm thick) were mounted on glass slides and stained with haematoxylin and eosin. Coded slides were examined by an experienced pathologist blinded to the treatment.

Statistical Analysis All quantitative data are expressed as mean±S.E.M. Comparisons between two groups were performed using Student's unpaired *t*-test, and comparison among groups more than two was carried out using one-way analysis of variance (ANOVA). Differences were considered to be significant when the degree of confidence in the significance was 95% of better ($p<0.05$).

RESULTS

Effect of *C. longa* on Gastric Acid Secretion and the Formation of Gastric Mucosal Lesions Using the Shay ulcer model, which causes gastric hypersecretion,[12] we investigated the effects of *C. longa* extract on gastric acid secretion and mucosal injury, and compared them with the effects of ranitidine. Ranitidine has been shown to have a marked anti-ulcer effect and has been used in the treatment and prevention of a variety of gastrointestinal disorders associated with gastric acid secretion.[13] Histopathological examination of gastric lesions showed discontinuation of the surface epithelium in the vehicle treated group. In contrast, *C. longa* extract protected the gastric mucosal layer as effectively as ranitidine (Fig. 1A). Using the pylori-ligated rat model, we found that *per os* administration of *C. longa* extract significantly inhibited gastric acid (Fig. 1D), gastric juice secretion (Fig. 1B), and ulcer formation (Fig. 1C), comparable to the effects of ranitidine. The ethanol extract of *C. longa* reduced the volume of gastric acid secreted after pylorus ligature, indicating that this may be the mechanism by which *C. longa* extract protects the gastric mucosa.

***C. longa* Extract Inhibits H_2 Histamine Receptor (H_2R)-Mediated Intracellular cAMP Production in U937 and HL-60 Promyelocytes** H_2R is the primary target of anti-ulcer drugs,[14] and H_2R antagonists have been shown to inhibit gastric acid secretion in many animal model systems.[13,15,16] H_2R is also expressed not only on gastric parietal cell but also on immune cells,[17] including the U937 and HL-60 promyelocytic cell lines, which have been widely used as model systems for H_2 histamine receptor activity.[9,18] We therefore tested the effect of dimaprit, a selective H_2R agonist, on the generation of cAMP in U937 and HL-60 cells. Application of various concentrations of dimaprit triggered cAMP production in a concentration-dependent manner (Fig. 2A), and with an EC_{50} of 5.5±0.9 μM. The effect of 10 μM dimaprit was inhibited by 10 μM of the specific H_2 receptor antagonist, ranitidine (10 μM), and by *C. longa* extract in a concentration-dependent manner (Fig. 2B, closed circle). In addition, *C. longa* extract also suppressed histamine-induced cAMP production in a concentration-dependent manner (Fig. 2B). Since *C. longa* extract may block Gsα-protein activity rather than H_2R, we tested the involvement of Gsα-protein in the activity of *C. longa* extract by treating the U937 cells with isoproterenol (500 nM), an agonist of the Gsα-protein coupled β_2-adrenoceptor, following treatment with *C. longa* extract.[19,20] *C. longa* did not affect isoproterenol-induced cAMP production (Fig. 2B, closed box), indicating that the

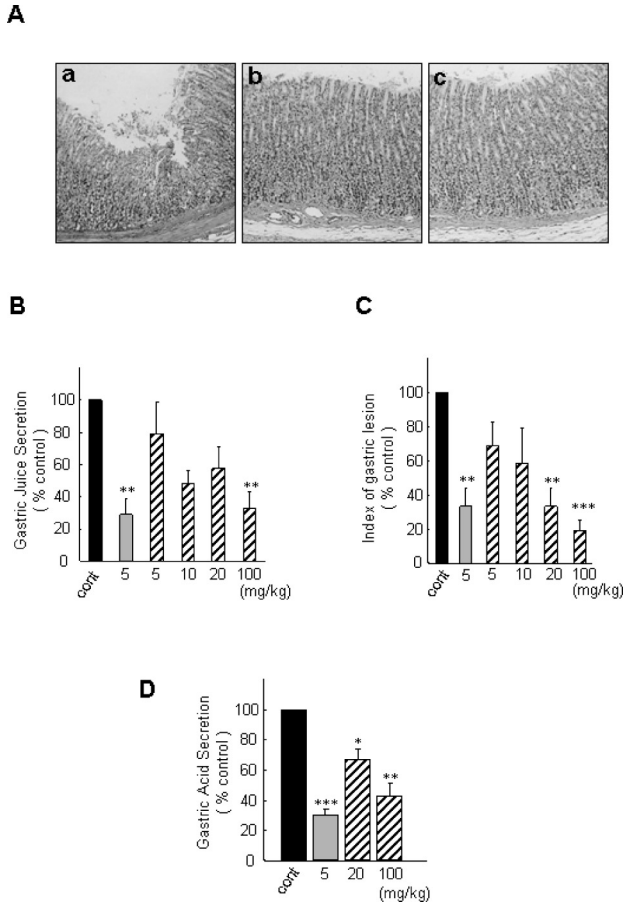

Fig. 1. Effect of *C. longa* Extract on Gastric Ulcer

(A) Microscopic appearance of pylori-ligation induced gastric lesions. The lesion formed in the gastric mucosa of vehicle treated (a), 5 mg/kg ranitidine treated (b), 100 mg/kg *C. longa* extract treated (c) rats. No mark infiltration of inflammatory cells was observed. Hematoxylin and eosin staining (original magnification: objective×100). (B) Effects of 80% ethanol extract of *C. longa* (stripe bar) and of ranitidine (gray bar) on the total gastric juice secretion after pylorus ligature in SD rats. (C) Protection effect of *C. longa* extract on the formation of gastric mucosal lesion. Each bar represents lesion score of Shay-model induced gastric damage. Test substances (vehicle (closed bar), ranitidine (gray bar), *C. longa* extract (stripe bar)) were injected through *per os* and then rats were killed 8 h after the surgery. (D) Effects of 80% ethanol extract of *C. longa* (stripe bar) and of ranitidine (gray bar) on the gastric acid secretion after pylorus ligature in SD rats. Each column represents the mean±S.E.M. (n=4—9 per group) and the difference between groups was determined by ANOVA. The marked column showed a significant difference in a comparison with the control: $*p<0.05$, $**p<0.01$ and $***p<0.001$.

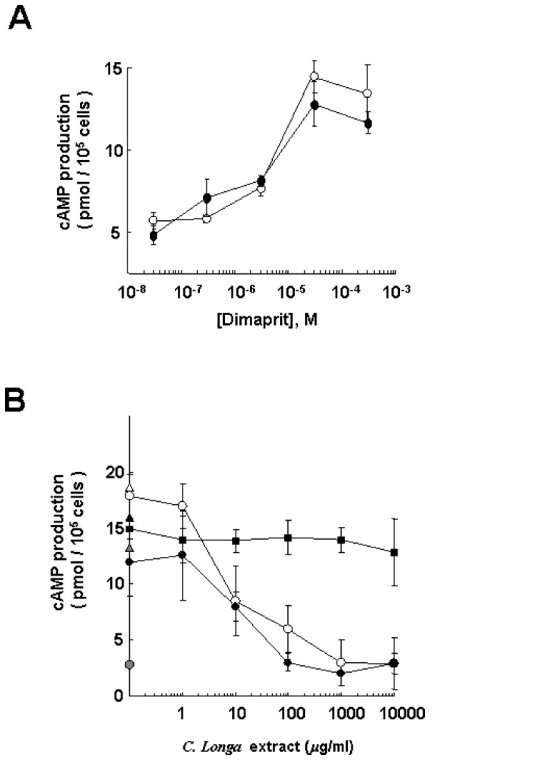

Fig. 2. Inhibition of H_2R-Mediated cAMP Production by *C. longa* Extract in U937 and HL-60 Cells

(A) Dimaprit increased intracellular cAMP in U937 (open circle) and HL-60 (closed circle) cells with concentration-dependent manner. cAMP generation was measured as described in Materials and Methods. (B) U937 Cells preincubated with 10 μM ranitidine (gray circle) or various concentrations of *C. longa* extract for 5 min, then cells were stimulated with 10 μM histamine (open circles) or dimaprit (closed circles). Ranitidine or *C. longa* extract was not removed. The effect of *C. longa* extract on the β_2-adrenoceptor was also tested with 500 nM isoproterenol treatment (closed box). Each triangle represents 10 μM histamine (open) 10 μM dimaprit (gray) and 500 nM isoproterenol (closed) treatment without *C. longa* extract, respectively. The results are the mean±S.E.M. of assay triplicates. Similar results were obtained in at least six independent experiments.

inhibitory effect of *C. longa* extract on the dimaprit-induced cAMP generation was through direct inhibition of H_2R. We could not detect significant cellular damage (by MTT assay) in cells treated with vehicle or pretreated with ranitidine or *C. longa* extract (data not shown), suggesting that the *C. longa* inhibition of dimaprit-induced cAMP responses was not due to cell death.

Effects of the Ethyl Acetate Fraction of *C. longa* and Curcumin on H_2R Signaling We subjected the 80% ethanol extract of *C. longa* to successive solvent extractions[21] and obtained the ethyl acetate (EA), *n*-buthanol (*n*-Buta), and water extracts. To determine the H_2R blocking activity of these extracts, U937 cells were pretreated with each extract, and the cells were subsequently treated with dimaprit. We found that both the EA and *n*-Buta extracts significantly inhibited dimaprit-induced cAMP production, but the effect of EA extract was more pronounced (Fig. 3A). Since the active constituent(s) may have accumulated in the EA extract, we compared the H_2R inhibitory activity of the EA and ethanol extracts of *C. longa*. We found that both extracts significantly inhibited H_2R in a concentration-dependent manner (Fig. 3B), with IC_{50}s of 6.0±0.5 μg/ml and 30.00±0.78 μg/ml, respectively. These findings indicate that substance in *C. longa* that acts H_2R is probably contained in the EA extract. Since curcumin is the major component of *C. longa*,[22] we investigated the effect of curcumin on dimaprit-induced H_2R signaling. Curcumin, however, showed no significant effect on H_2R activity (Fig. 3C). Curcumin alone has no effect on cAMP formation (Fig. 3C).

[^{3}H]-Tiotidine Binding Assay with *C. longa* Extract To determine whether the effects of *C. longa* extract were due to its ability to block histamine binding to H_2R, we tested the effects of *C. longa* extract on [^{3}H]-tiotidine binding to H_2R in undifferentiated HL-60 cells.[9] We found that both ranitidine and *C. longa* ethanol and EA extract significantly blocked [^{3}H]-tiotidine binding to undifferentiated HL-60 cells (Fig. 4), and 10-fold low concentration of EA extract showed more potency rather than ethanol extract.

DISCUSSION

C. longa has been widely used as anti-ulcer remedy in tra-

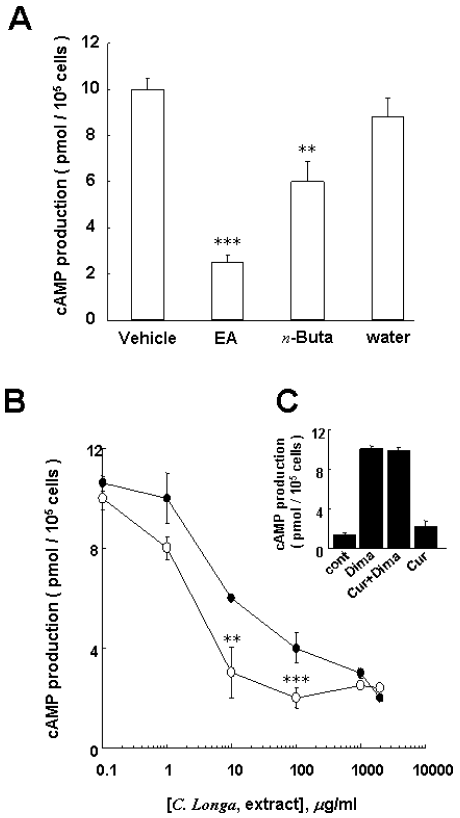

Fig. 3. (A) Effect of *C. longa* Ethyl Acetate (EA), *n*-Butanol (*n*-Buta) and Remaining Water Extract (0.1 mg/ml Each) on 10 μM Dimaprit-Induced cAMP Production in U937 Cells

The marked column showed a significant difference in a comparison with the vehicle (10 μM dimaprit alone) : $^{**}p<0.01$ and $^{***}p<0.001$.

(B) Dose–Response Curves of *C. longa* Ethanol Extract (Closed Circle) and Its EA Fraction (Open Circle) on H_2R Mediated cAMP Production

Each point is the mean±S.E.M. of triple experiments. The difference between groups was determined by ANOVA. The marked column showed a significant difference in a comparison with the ethanol extract: $^{**}p<0.01$ and $^{***}p<0.001$.

(C) The Effect of 1 μM of Curcumin (Cur) on 10 μM Dimaprit (Dima)-Induced cAMP Production in U937 Cells

Each point is the mean±S.E.M. of triple experiments.

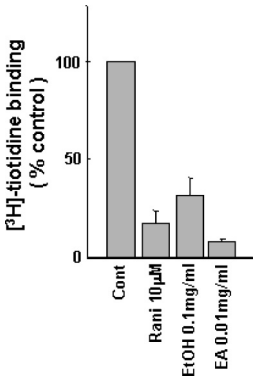

Fig. 4 Binding of [^{3}H]-Tiotidine to HL-60 Cells

Cells preincubated with indicated concentration of ranitidine (Rani), *C. longa* ethanol extract (EtOH), or *C. longa* ethylacetate fraction (EA) for 10 min were treated with [^{3}H]-tiotidine at 4 °C for 80 min. Data were calculated as the mean±S.E.M. of assay triplicates. Similar results were obtained in at least three independent experiments. The difference between groups was determined by ANOVA.

ditional medicine,[3,4] but its action mechanism is still unclear. In the present study, we have provided a possibility that the *C. longa* ethanol extract prevent the development of gastric ulcers by blocking H_2R.

The *C. longa* extract induced inhibition of cAMP signaling is due to blocking of histamine binding to H_2R, rather than acceleration of degradation, since the inhibition was observed in the presence of the phosphodiesterase inhibitor Ro20-1724. In addition, the inability of *C. longa* extract to inhibit β_2-adrenoceptor mediated cAMP production (Fig. 2B) indicates that the active component(s) of *C. longa* extract are highly selective, inhibiting only the binding of histamine (or dimaprit) to H_2R (Fig. 4). In addition, EA fraction of *C. longa* extract showed prominent H_2R antagonistic effect rather than other fractions, suggesting that specific activity was increased (about 4-fold) by successive extraction with EA (Fig. 3B), indicating that further fractionations and purifications of EA fraction will give a possibility to find active single compound more potent that ranitidine.

In previous report showed that the *C. longa* extract has curcumin, demethoxycurcumin, bisdemethoxycurcumin, and ar-turmerone.[22] Among these, the curcumin is the major component of *C. longa* and purified curcumin was more active than either demethoxy- or bisdemethoxycurcumin.[22] In our study, however, pure curcumin showed no significant effect on dimaprit-induced H_2R signaling in our system (Fig. 3C). These results demonstrate that unidentified minor constituent in *C. longa* extract might be associated with H_2R blocking. There are two previous reports about the constituents in the *C. longa* EA extract.[23,24] In these studies, curcumin I (1), curcumin II (2), and curcumin III (3) were isolated as active principles in the EA extract. Therefore, further experiment is required whether these compounds are related with H_2R regulation and gastric acid secretion. This will help us to determine the critical compound in *C. longa* extract related with H_2R blocking and anti-ulcer effect.

Antagonism of H_2R has been the cornerstone of an immense market for pharmacological treatments of acid-peptic disorders of the gastrointestinal tract.[14] The selective inhibition by *C. longa* of H_2R and gastric acid secretion indicate that *C. longa* extract or its active component(s) may be promising therapeutic candidates for the treatment of gastric ulcers and other H_2R related diseases.[25]

H_2R antagonists have widely been used for the treatment of gastric-ulcer. Despite H_2R antagonists are relatively safe drugs, cimetidine, however, is known to interfere with other drugs through hepatic metabolism, the concentration of co-committant drugs such as warfarin, benzodiazepine tend to be elevated.[26] Nevertheless further experiments are required, we expect that *C. longa* extract or its active component(s) may replace and reduce the cimetidine related side effects.

In conclusion, our data suggest that *C. longa* extract shows selective and competitive H_2R antagonistic effects, indicating a mechanism for the reduction in gastric ulceration observed for *C. longa* extract. By blocking H_2R, this extract would provide another remedy for the treatment of gastric ulcers.

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