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The anti-inflammatory potential of turmeric-added Thai curry pastes in a simulated inflamed intestinal epithelium

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ABSTRACT: Thai curry pastes have been studied for their medicinal advantages due to their herb composition, yet research specifically on the gut health benefits of Thai curry paste remains limited. The objective of this study was to investigate the anti-inflammatory effects of both original Thai curry pastes and those with added turmeric in lipopolysaccharide (LPS)-activated macrophages and an intestinal inflammation model. Both southern Thai sour curry paste (MS) and Thai red curry paste (MR) were supplemented with four times the amount of turmeric used in the original formula and were digested *in vitro* before examination. The inflammatory biomarkers determined in inflamed cell models were nitric oxide (NO), inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), interleukin-8 (IL-8), and transepithelial electrical resistance value (TEER). The study demonstrates that the MS and MR pastes had potentially inhibitory effects on NO and TNF-α compared to the original samples. Only the MS paste inhibited the expression of iNOS and the pathogenesis of macrophage-induced intestinal epithelial inflammation, as evidenced by an increase in TEER and a decrease in IL-8. The study finds that diminishing macrophage cell activity in modified southern sour Thai curry paste with four times more turmeric can mitigate intestinal epithelial inflammation. This research could influence the development of Thai curry paste-based herbal nutraceuticals for gastrointestinal health.

KEYWORDS: Thai curry pastes, inflamed intestinal epithelium, LPS-stimulated RAW264.7, *Curcuma longa* L.

INTRODUCTION

Inflammatory bowel diseases (IBD), the most common forms of which are Crohn's disease and ulcerative colitis, are recurrent inflammatory illnesses that affect the gastrointestinal tract on a chronic basis. IBD is a global healthcare issue with steadily increasing incidence in many regions, including countries in Asia [1–3]. Dietary and lifestyle changes may have a role in the pathogenesis of IBD, which is caused by a combination of environmental, genetic, microbiome, and immune factors. This phenomenon can disrupt the homeostasis of the intestines, ultimately resulting in an onset of pathology and disease [4, 5].

Macrophages in the intestine play a crucial role in the pathogenesis of intestinal inflammation. When intestinal homeostasis is disrupted, pathogens are able to pass through the damaged intestinal epithelial cell barrier and stimulate M1 macrophages to produce significant quantities of TNF-α and NO. These proinflammatory mediators disrupt the integrity of the epithelial cell layer by causing cell death, which is the initial stage in the process of intestinal inflammation [6, 7]. In the context of inflammatory processes, the intestinal mucosa has been observed to release a chemokine called IL-8 at the site of inflammation. The chemokine has been identified as the mediator responsible for the recruitment and transmigration of neutrophils into inflamed tissues. This process subsequently promotes inflammatory response and tissue damage [8, 9]. Modulating the pathways involved in the specification of intestinal macrophages could therefore lead to a strategy for the prevention and treatment of IBD.

Current treatments for IBD involve the use of immunosuppressants and steroids, which lessen the impact of inflammation; nevertheless, these drugs come with unwanted side effects. To avoid negative reactions and reduce therapeutic costs, natural therapies have been suggested because they are associated with fewer adverse effects than conventional methods. Preclinical and pilot study research provide evidence supporting the effectiveness of natural anti-inflammatory agents in preventing and treating inflammatory IBD. Consequently, there has been a significant amount of attention given to exploring potential natural products for the prevention and treatment of IBD and its associ-

Table 1 Ingredients of the 4 recipes of the southern Thai curry pastes.

Ingredient (%)	OS	MS	OR	MR
Fresh red chili (*Dendranthema indicum* L.)	59.5	52.0	–	–
Dried red chili (*Dendranthema indicum* L.)	–	–	21.3	18.4
Shallot bulb (*Allium ascalonicum* L.)	16.7	14.6	21.3	18.4
Garlic bulb (*Allium sativum* L.)	19.0	16.7	16.0	13.8
Turmeric (*Curcuma longa* L.)	4.8	16.7	5.3	18.4
Fresh lemon grass (*Cymbopogon citrates* L.)	–	–	21.3	18.4
Fresh galangal (*Alpinia galangal* L.)	–	–	5.3	4.6
Kafferlime peel (*Citrus hystrix* DC.)	–	–	1.3	1.1
Dried pepper seed (*Piper nigrum* L.)	–	–	8.0	6.9

ated conditions [5, 10, 11].

Thai curry paste is a basic component in Thai cuisine and results in Thai food's aromatic and flavorful characteristics. Thai curry paste typically includes a blend of aromatic Thai herbs and spices such as lemongrass, kaffir lime leaves, shallots, garlic, chilies, and turmeric. Several Thai curry pastes have been shown to contain highly bioactive compounds and exhibit interesting antioxidant activities and anti-inflammatory effects on LPS-activated macrophages by reducing the production of pro-inflammatory mediators [12–16]. Various Thai curry pastes contain turmeric (*Curcuma longa* L.), a notable herbaceous component. Turmeric has been historically utilized to treat inflammatory conditions of the digestive tract and other organs. Despite identification of the main chemical components—curcumin, demethoxycurcumin, and bisdemethoxycurcumin—these compounds remain unsuitable for pharmaceutical applications due to their low bioavailability and instability. Consequently, they are instead frequently employed in the form of dietary supplements.

Although research has shown that turmeric can reduce inflammation in the digestive tract [17], research on Thai curry paste containing turmeric in gastrointestinal inflammation models is limited, resulting in a lack of scientific evidence demonstrating whether Thai curry paste is effective in preventing and relieving digestive inflammation. The objective of this study was to investigate the anti-inflammatory effects of original and turmeric-added Thai curry pastes, sour curry paste, and red curry paste in LPS-stimulated RAW264.7 macrophages and in a co-cultured model made up of Caco-2 monolayers (mimicking the intestinal barrier) and RAW264.7 macrophages. The findings of this research add to existing knowledge and can support the development of Thai curry paste-based herbal nutraceuticals to improve gastrointestinal health.

MATERIALS AND METHODS

Preparation of the curry paste

In this investigation, a southern Thai sour curry paste known as Kaeng-Som and a southern Thai red curry paste known as Kaeng-phed were selected for study. The turmeric rhizome was obtained from Bantakhun Village in Surat Thani Province, while the other ingredients were sourced from Plaza Market in Hat Yai, Songkhla Province. As shown in Table 1, 4 formulations were created: the original sour curry paste (OS); the modified sour curry paste (MS); the original red curry paste (OR); and the modified red curry paste (MR). All materials were grounded using a blender (Panasonic, MX 151 SP, Japan), sieved through a 40 mm mesh, labeled, collected in zip-locked bags, and stored at −20 °C. Samples of 250 g each were freeze-dried (Dura Freeze Dryer, Canada) to obtain about 50 g of solids. The resulting powder samples were stored in zip bags and stored at −20 °C for 2 days before digestion in simulated conditions.

In vitro digestion of the curry paste

The *in vitro* digestion of the curry pastes (OR, OS, MR, and MS) was performed consecutively, simulating the physiological digestive process as described by Le Roux et al [18] and Nguyen et al [19]. Each sample underwent stepwise digestion, passing through the oral, stomach, and intestinal phases sequentially to mimic human digestion conditions as follows:

Simulated oral digestion: 30 g of curry paste was mixed with 300 ml of artificial saliva (the Faculty of Dentistry, Prince of Songkla University Thailand), pH 6.8, with α-amylase, final concentration of 0.33 units/ml amylase, stirred and incubated at 150 rpm at 37 °C for 5 min. Consequently, the samples were boiled at 100 °C for 15 min to stop the enzyme activity and then cooled down.

Simulated digestion in the stomach: The samples from the oral phase were further digested in simulated gastric fluid (without enzyme) at a ratio of 1:2, where 1 part of the sample was mixed with 2 parts of simulated gastric fluid. The pH was then adjusted to 2 using 6 N HCl, then pepsin (P7000 from porcine gastric mucosa, Sigma-Aldrich, USA) was added to a final concentration of 22.75 unit/mg of protein. The samples were stirred and incubated at 150 rpm, 37 °C for 2 h. The samples were boiled at 100 °C for 15 min to stop the enzyme function and cool down quickly.

Simulated digestion in the small intestine: The samples were further digested by adding simulated intestinal fluid (without enzyme) in a 1:2 ratio to the initial sample in the mouth. The pH was adjusted to 6.8 with 6 N NaOH. At the specified pH, bile salt was added to a final concentration of 2 mM, and pancreatin was added to a final concentration of 10 mg/ml. They were incubated at a speed of 150 rpm, a temperature of 37 °C for 4 h. The enzyme inactivation was achieved by heating at 100 °C for 25 min and cooling rapidly by placing on ice. The digested samples were removed from sugars, amino acids, and fatty acids by dialysis using a dialysis bag containing 1 KDa MWCO. The dialysis time was 24 h in cold distilled water. The

samples in the retentate section in the bag were freeze-dried. The freeze-dried powder was then stored in a zipper bag and stored at −20 °C for further testing in a human intestinal model.

Cell lines and cell culture reagents and materials

RAW264.7 cell line was purchased from ATCC (Manassas, USA). Caco-2 cell line was purchased from CLS Cell line service (GmbH, Germany). All cell culture media and supplementary reagents were purchased from Gibco, Life Technology (Carlsbad, USA). All cell culture materials were purchased from SPL Life Science (Gyeonggi-do, Korea). Transwell permeable support with 0.4 μm pore polycarbonate membrane was purchased from Corning (Corning, USA).

Chemicals

LPS from *Escherichia coli* O111:B4, dimethyl sulfoxide (DMSO), dexamethasone (Dex), Griess reagent (modified), curcumin (CAS 458-37-7) were purchased from Sigma-Aldrich. Enzyme-linked immunosorbent assay (ELISA) kits were obtained from BioLegend (San Diego, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Invitrogen, Life Technologies (Carlsbad, USA). Chemicals for SDS-PAGE and protein sample preparation were purchased from Bio-Rad (Hercules, USA). Primary and secondary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, USA). Luminita™ Chemiluminescent, HRP substrate, was purchased from EMD Millipore (Billerica, USA). Hyperfilm™ ECL™ film was purchased from Cytiva (Marlborough, USA).

Cell culture

RAW264.7 and Caco-2 cell lines were cultured in sterile conditions in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were maintained in a CO_2 incubator with 5% CO_2 and 95% humidity at 37 °C. Cell culture media were changed every 2 days in a sterile manner. The cells were subcultured when confluency was reached to 80% using 1:4 split ratio.

Cytotoxicity test

RAW 264.7 cells were seeded in 96-well plates at a density of 1.0×10^4 cells per well. The cells were grown for 48 h. OS, MS, OR, and MR were diluted in complete DMEM to obtain a final concentration at 5.0 mg/ml and then serially diluted to 0.156 mg/ml. The cells were treated with samples for 1 h, followed by LPS activation for 24 h. After the 24 h-treatment, the spent media were removed, and 200 μl of 0.5 mg/ml MTT solution was added to each well and incubated for 4 h. The formazan crystals were dissolved in DMSO. The absorbance (Abs) was detected at 560 nm, and background at 670 nm was subtracted, using a microplate reader. Cell viability was calculated as follows:

$$\% \text{ Cell viability} = \frac{\text{Abs. treated}}{\text{Abs. untreated}} \times 100.$$

Nitric oxide assay

Nitrite assay for measuring NO degradation in RAW264.7 macrophage cells was determined by Griess reagent reaction. Cells were pre-incubated with various concentrations of OS, MS, OR, and MR and Dex as a standard drug for 1 h before 10 ng/ml LPS stimulation. After 24-h incubation, 50 μl of supernatants were collected and mixed with 50 μl of Griess reagent (1% w/v) in a 96-well plate. After 30 min of incubation at RT, absorbance was measured at 540 nm using a microplate reader. NO production was proportional to the content of nitrite level. Concentration of nitrite was determined using a $NaNO_2$ standard curve with $r^2 > 0.99$.

Determination of half maximal inhibitory concentration (IC_{50}) and selectivity index (SI)

IC_{50} was determined from a dose response curve (inhibitory effect). SI was calculated by comparing IC_{50} values for the cytotoxicity in RAW264.7 against the IC_{50} of the same sample in a NO inhibition.

Co-culture for determination of gut inflammation condition

Caco-2 cells were seeded into 12-well Transwell insert plates at 0.5×10^5 cells/well. The cell culture medium was changed every 2–3 days for 21 days. RAW264.7 cells were seeded on the basolateral side of the Transwell at a density of 1×10^5 cells/well and incubated for 48 h to facilitate complete adherence to the well. After 48 h, all media were replaced with DMEM. To assess the anti-inflammatory activities of samples in this model, samples were treated on the apical side with MS and MR at concentrations ranging from 0.6–2.5 mg/ml for 2 h. After incubation, the RAW264.7 cells were stimulated by the addition of 10 ng/ml of LPS to the basolateral side of the co-culture models. After an additional 22-h incubation, TEER values were measured. Both apical and basolateral culture media were collected for measurement of cytokine levels.

TEER measurement

All experiments were performed on differentiated Caco-2 cell monolayers, 21 days post-seeding. In order to check the integrity of the Caco-2 monolayer, TEER was measured by employing the Millicell Electrical Resistance System from Millipore (Bedford, USA). Only monolayers with TEER values beyond 400 $\Omega \times cm^2$ were used in the experiments.

Enzyme-linked immunosorbent assay

Cell-free culture supernatants of RAW264.7 or Caco-2 were collected and subjected to ELISA of TNF-α and IL-8 using commercial ELISA assay kits, according to the manufacturer's instructions.

Western blot analysis

RAW 264.7 cells were plated on 6-well plates at 3.0×10^5 cell/cm^2. After 24-h incubation, cells were pre-treated with various concentrations of MS and MR for 1 h, then stimulated with LPS (10 ng/ml) for 18 h for iNOS study. Cell pellets were lysed in a lysis buffer containing a protease inhibitor cocktail. Supernatants from the cell lysates were collected by cold centrifugation at 14,000 *g* for 10 min. Protein concentrations from whole cell lysates were measured using a Detergent compatible protein assay kit. Protein samples were prepared by mixing 30 µg of protein with 2 × Laemmli sample buffer and heated at 95 °C for 5 min. The proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. After subsequent blocking with 5% non-fat dry milk, the membranes were incubated overnight at 4 °C with primary antibodies iNOS (1:1000) and β-actin (1:5000). The membranes were washed in a mixture of Tris-buffered saline and Tween-20 (TBST) and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) for 1 h. The expression of protein bands was visualized by chemiluminescent reaction using hyperfilm detection.

Analysis of curcumin using High Pressure Liquid Chromatograph (HPLC)

An examination of curcumin was carried out according to an adapted method [20] using an HPLC system (Agilent Model 1200 series, Germany). The system included a diode-array detector (DAD) and was outfitted with an Agilent Eclipse Plus C18, 4.6*250 mm, 5 µm column (Restek, Bellefonte, USA). The mobile phase was composed of 75% (v/v) methanol and had a flow rate of 1 ml/min. The signal was detected at a wavelength of 419 nm. The curcumin content was measured using standard curves, with a linearity of R^2 greater than 0.99.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) from at least 3 independent experiments. Statistical comparisons were performed using Student's *t*-test for differences between 2 groups and by one-way ANOVA with Dunnett's post hoc test for differences among multiple groups. Significance was assigned for *p* values < 0.05 using GraphPad Prism 5.0 (GraphPad Software, USA).

RESULTS

The effect of digestates on nitric oxide inhibition in LPS-activated murine macrophages

In vitro digestates of 4 different formulas of southern Thai curry pastes (OS, MS, OR, and MR) (details shown in Table 1) were evaluated for toxicity and nitric oxide inhibitory activities in RAW264.7 macrophages. It was found that the 4 digestates were non-toxic to macrophages at concentrations ranging from 0.15 to 2.50 mg/ml (Fig. 1A,B). However, the cytotoxic effects of modified formulations MS and MR at a concentration of 5.0 mg/ml on macrophages were observed. Particularly, MS exhibited a remarkable cytotoxic effect, leading to a 95.2% reduction in cell viability (Fig. 1A). Similarly, MR also displayed a substantial cytotoxic effect, resulting in a 67.6% decrease in cell viability (Fig. 1B) ($^{\&}, p < 0.05$ vs. 0 mg/ml). The anti-inflammatory effect of nitric oxide levels in LPS-stimulated RAW264.7 cells showed that both MS and MR, when present at concentrations exceeding 1.25 mg/ml, exhibited a notable reduction in nitric oxide levels compared to the LPS group (Fig. 1C,D). MS and MR at concentration of 2.5 mg/ml were found to be the most effective to reduce NO levels without causing any cytotoxic effects. The results showed that MS reduced NO levels by 68.2% (Fig. 1C), while MR reduced NO levels by 69.5% (Fig. 1D) ($^{*}, p < 0.05$ vs. LPS). Furthermore, the anti-inflammatory effect of MS and MR was found to be significantly stronger than that of OS and OR ($^{\#}, p < 0.05$). These findings suggest that both MS and MR have potential as NO inhibitors, with the concentration of 2.5 mg/ml being the most optimal to achieve significant reductions in NO levels.

To assess the potential of the digestates, the concentrations at which 50% cytotoxicity (CC_{50}) and IC_{50} occurred were calculated. A low IC_{50} indicates that the digestate effectively inhibits nitric oxide production at a low concentration, suggesting strong anti-inflammatory potential. A high CC_{50} value reflects low cytotoxicity, meaning the digestate is safe for cells at higher concentrations. The relationship between these 2 values is expressed as SI, calculated as CC_{50}/IC_{50}. A higher SI value indicates a better balance between efficacy and safety, meaning the digestate can reduce inflammation with a low cytotoxicity. SI values are often used for evaluating any anti-inflammatory and anti-cancer activities of medicinal substance *in vitro*. In the present study, the IC_{50}, CC_{50}, and SI values obtained from the 4 distinct formulations were compared, as shown in Table 2. The MS and MR had IC_{50} of 1.63 and 1.75, respectively, which were considerably lower than those of the original OS and OR. The SI values of the MS and MR were better than those of the original formula, which was consistent with the IC_{50} values. In addition, the anti-inflammatory activity of Dex, a type of corticosteroid, was investigated as a reference. Dex was used in the nitric oxide detection

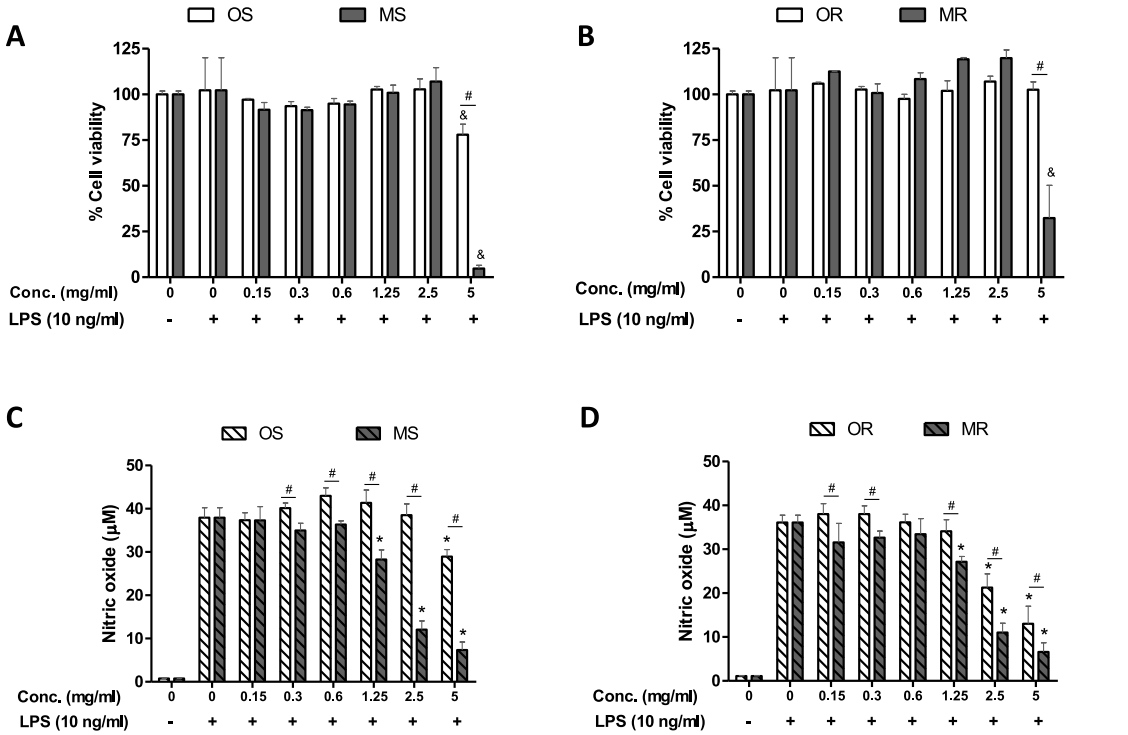

Fig. 1 Effects of the 4 digested curry pastes on cell viability and NO production in LPS-activated RAW 264.7 cells. RAW 264.7 cells were pretreated with the OS, MS, OR, and MR at various concentrations (0.15–5.00 mg/ml) for 1 h before LPS (10 ng/ml) stimulation for 24 h. (A, B) Cell viability of the sour yellow curry paste (OS and MS) and hot curry paste (OR and MR) determined by the MTT assay. (C, D) Nitrite levels upon LPS stimulation with and without the digestates measured by the Griess assay. The results are expressed as the mean ± SD of 3 independent experiments in triplicate. $^{\&}p < 0.05$ vs. control group without LPS stimulation; $^{*}p < 0.05$ vs. LPS-stimulated group; $^{\#}p < 0.05$ vs. original formula-treated group.

Table 2 50% cytotoxicity concentrations (CC_{50}), 50% nitric oxide-inhibitory concentrations (IC_{50}), and selectivity index (SI) of digestates of 4 different formulations in LPS-activated macrophages.

Sample	Code	CC_{50} (mg/ml)	IC_{50} (mg/ml)	SI
Digestates	OS	> 7.0	> 5.0	–
	MS	3.89 ± 0.06	1.63 ± 0.14	2.4
	OR	> 7.0	2.17 ± 0.10	3.2
	MR	4.54 ± 0.44	1.75 ± 0.11	2.6
Reference	Dex	> 0.16	0.078 ± 0.004	> 2.0

Dex = Dexamethasone.
SI = Selectivity index (CC_{50}/IC_{50}).

assay as a positive control to validate the inflammatory response of RAW264.7 cells. According to the present study, Dex exhibited a SI value of 2.0, which is lower than that observed in the tested curry pastes. These findings suggest that the modified curry pastes are better able to reduce inflammation.

The effect of digestates on pro-inflammatory cytokine production

In IBD, TNF-α is a type of protein generated by macrophage cells when they are in inflammatory condition. In the present study, it was observed that the administration of MS and MR at a concentration of 0.625 mg/ml exhibited notable efficacy in diminishing the production of TNF-α induced by LPS. However, increasing the concentration did not have any effect on the TNF levels (Fig. 2A,B).

The effect of digestates on iNOS protein suppression

The iNOS is significant in the process of NO synthesis. To gain a deeper understanding of the anti-inflammatory mechanism in macrophages, this study also investigated the impact of MS and MR on the expression of iNOS. From the results in Fig. 3, there is a significant increase in the expression of intracellular iNOS protein bands upon exposure to LPS. This increase was found to be statistically significant when compared to the control group ($p < 0.05$). Our findings indicate that MR at concentrations of 0.63–2.50 mg/ml did not result in a statistically significant decrease in iNOS levels within the treated cells. The treatment with MS at concentrations of 1.25 and 2.50 mg/ml led to a significant decrease in iNOS protein expression compared to cells treated with

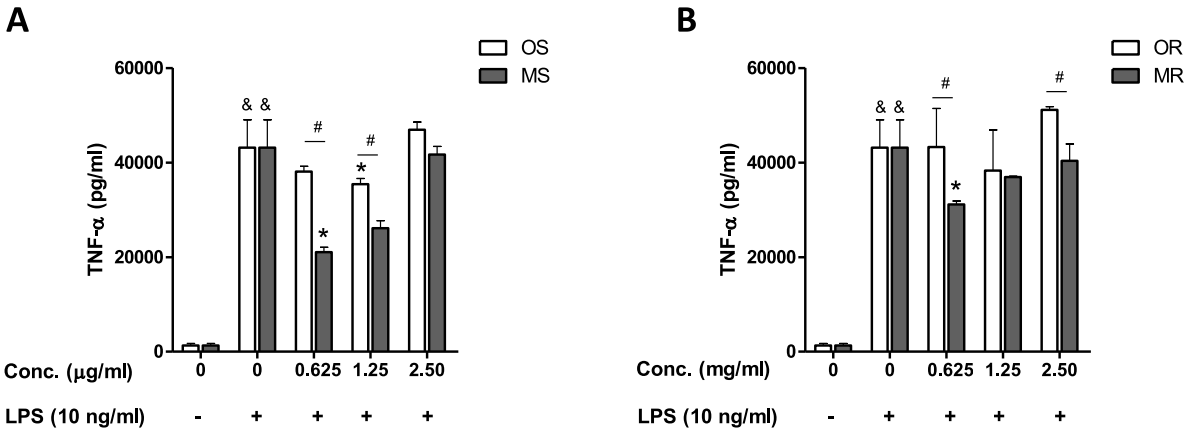

Fig. 2 Effects of the 4 digested curry pastes on TNF-α levels in LPS-activated RAW 264.7 cells. RAW 264.7 cells were pretreated with the OS, MS, OR, and MR at various concentrations (0.625–2.50 mg/ml) for 1 h before LPS (10 ng/ml) stimulation for 24 h. TNF-α levels of the sour yellow curry paste (OS and MS) A) and hot curry paste (OR and MR) (B) determined by ELISA. The results are expressed as the mean ± SD ($n = 3$). $^{\&}p < 0.05$ vs. control group without LPS stimulation; $^{*}p < 0.05$ vs. LPS-stimulated group; $^{\#}p < 0.05$ vs. original formula-treated group.

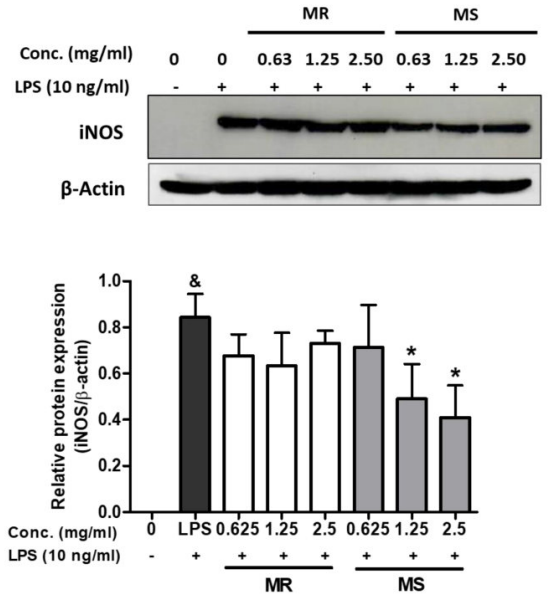

Fig. 3 Effects of the digested modified curry pastes MS and MR on iNOS expression in LPS-activated RAW 264.7 cells. RAW 264.7 cells were pretreated with the OS, MS, OR, and MR at various concentrations (0.625–2.50 mg/ml) for 1 h before LPS (10 ng/ml) stimulation for 24 h. Protein expression of iNOS and β-actin (internal control) was determined by Western blot analysis using corresponding antibodies (upper panel). Relative expression levels of the proteins were determined by densitometric analysis (lower panel). Each value indicates the mean ± SD ($n = 3$). $^{\&}p < 0.05$ vs. control group without LPS stimulation; $^{*}p < 0.05$ vs. LPS-stimulated group.

10 ng/ml LPS ($^{*}, p < 0.05$).

Anti-inflammatory effects of digestates in the co-culture of intestinal Caco-2 and macrophages

To evaluate the potential anti-inflammatory effects of MS and MR on the intestines, a co-culture model was established. This model involved the use of intestinal epithelial Caco-2 cells on the apical side and LPS-stimulated macrophage RAW264.7 cells on the basolateral side. In this study, the measurement of transepithelial electrical resistance (TEER), a widely recognized marker of epithelial integrity, was performed. As shown in Fig. 4A, we observed a significant decrease in TEER following the stimulation of RAW264.7 cells with LPS. The decrease in TEER amounted to 55.05% and was determined to be statistically significant ($^{\&}, p < 0.05$). Treatment with MS at a concentration of 0.625 mg/ml significantly preserved the integrity of the Caco-2 cell monolayers. However, the higher concentrations of MS did not demonstrate any protective effect in our experimental model, indicating the potential protective effects of different concentrations of MS on a specific condition. Additionally, treatment with MR was found to be ineffective in protecting against the condition of interest (Fig. 4A).

IL-8 is generated by intestinal epithelial cells. When basolateral intestinal epithelial cells are destroyed by pro-inflammatory chemicals such as TNF-α and NO produced by macrophages, a secretory response is induced. Herein, the study of IL-8 was aimed at elucidating the mechanism of how MR and MS protect intestinal epithelial cells. The result showed that LPS stimulation significantly increased IL-8 secretion in the intestinal mucosa ($^{\&}, p < 0.05$ vs. 0 ng/ml LPS). At a concentration of 0.625 mg/ml, MS was able to diminish IL-8 from inflammation induction,

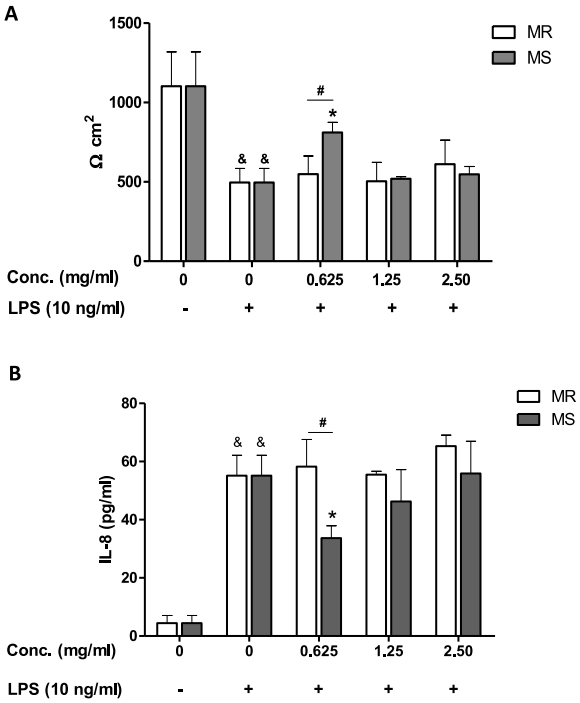

Fig. 4 Effects of the digested modified curry pastes MS and MR on protection against macrophage-induced Caco-2 intestinal monolayer dysfunction. Caco-2 were differentiated for 21-days on apical side and co-cultured with RAW264.7 on the basolateral side of the Transwell. The caco-2 were then pretreated with the MS and MR at various concentrations (0.625–2.50 mg/ml) for 2 h before LPS (10 ng/ml) stimulation for 22 h. (A) TEER determined by Millicell Electrical Resistance System. (B) IL-8 in culture media from apical side determined by sandwich-ELISA. Each value indicates the mean ± SD ($n = 3$–6). (C) IL-8 in culture media from basolateral side upon treatment with MR and MS at 0.625 mg/ml determined by sandwich-ELISA. Each value indicates the mean ± SD ($n = 4$–6). For (B) and (C), $^{\&}p < 0.05$ vs. control group without LPS stimulation; $^{*}p < 0.05$ vs. LPS-stimulated group; $^{\#}p < 0.05$ between MS and MR. (D) Cell viability of the Caco-2 cell monolayer upon 24 h treatment with MS and MR (0.31–5.0 mg/ml) determined by MTT assay. Values are expressed as mean ± SD ($n = 3$–4).

which was statistically significant ($^{*}, p < 0.05$ vs. LPS 10 ng/ml) (Fig. 4B) and consistent with the improved TEER effects (Fig. 4A). However, MS at concentrations of 1.25 and 2.50 mg/ml as well as MR at any concentration were unable to lower IL-8 levels (Fig. 4B). Because IL-8 can pass through the pore size into the basolateral chamber, we investigated IL-8 levels on the basolateral side after treatment with 0.625 mg/ml of MR and MS under the same treatment conditions. As demonstrated in Fig. 4C, IL-8 appeared in the basolateral compartment, particularly after LPS stimulation. Interestingly, IL-8 levels reduced significantly after MS treatment in comparison to LPS and MR, indicating that MS repressed IL-8 production. In addition, no cytotoxicity towards Caco-2 cell monolayers was observed (Fig. 4D).

Curcumin content in the MS digestates

The passage of curcumin through the intestinal epithelial cell model of MS was assessed by quantifying the quantity of curcumin in the cell culture medium collected from the apical and basolateral sides of the cell model after incubation with MS at a concentration of 0.625 mg/ml for a duration of 2 h. Prior to incubation, the initial concentration of curcumin in MS (at a concentration of 0.625 mg/ml) was measured to be 0.3556 ± 0.0268 µg/ml (Fig. 5B). After the incubation period, the concentration of remaining curcumin on the apical side was measured to be 0.2122 ± 0.006 µg/ml (Fig. 5C). However, curcumin was not identified in the sample taken from the basolateral side (Fig. 5D) because it was below the detection limit.

DISCUSSION

Although previous studies have reported the anti-inflammatory effect of Thai curry paste extract on macrophages [12, 16], limited research has been conducted on the anti-inflammatory activity of the curry pastes, particularly in their digested state. The present study investigated the retention of anti-inflammatory properties in macrophages following digestion of modified curry pastes. The findings reveal that even after undergoing the digestion process, the modified curry pastes exhibited sustained anti-inflammatory effects in macrophages. A previous study examined the hydrolysis rate of curry paste in various biological environments, including saliva, gastric juice with a pH of 2.0, and intestinal enzyme (specifically, pancreatic amylase). Cumulatively, these hydrolysis processes accounted for approximately 12% of the total weight of the curry paste. Therefore, subsequent to the ingestion of the curry paste, it is probable that approximately 88% of the curry paste will travel into the colon. The observed phenomenon can be attributed to the composition of Thai curry paste, which reportedly contains relatively low quantities of crude protein, crude fat, and dietary fiber [21].

In the present study, the original and modified southern Thai curry pastes (sour and red curry pastes) were digested before being evaluated for anti-inflammatory activity in LPS-stimulated macrophages, as well as in co-cultures of intestinal epithelial cells with macrophages. The results of these investigations showed that the original and modified southern Thai curry pastes had different levels of biological activity. The obtained results clearly indicate that the addition of turmeric in the modified formulation led to significantly higher anti-inflammatory efficacy compared to the original formulations. This was evidenced by

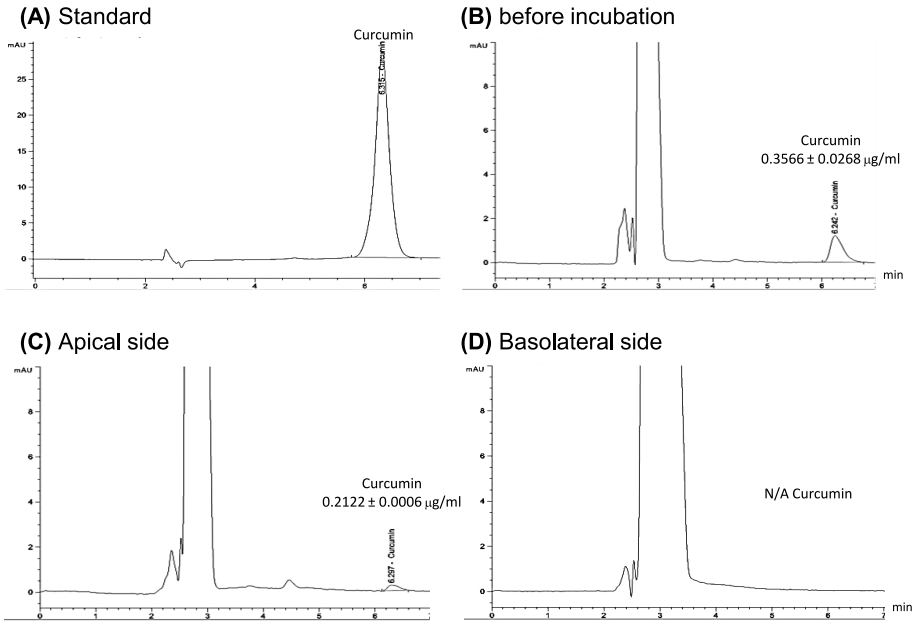

Fig. 5 HPLC chromatograms of (A) pure curcumin, (B) curcumin in the cell culture medium mixed with MS at 0.625 mg/ml, (C) curcumin in the cell culture medium from the apical side after the incubation with MS for 2 h, and (D) curcumin in the cell culture medium from the basolateral side after the incubation with MS for 2 h.

a notable reduction in the levels of NO and TNF-α, suggesting the potential of turmeric as a valuable ingredient to combat inflammation. The observed phenomenon can be attributed to the unique composition of curcuminoids, which includes bisdemethoxycurcumin as the primary bioactive compound responsible for the targeted anti-inflammatory properties of turmeric [22, 23]. Compared to MR, MS had a greater quantity of turmeric, which led to the production of the most potent anti-inflammatory substance in this study. Four times the amount of turmeric as in the original formula was used, because this concentration resulted in the highest antioxidant activity without negatively affecting the taste of the product. Additionally, the present investigation suggests that the anti-inflammatory properties exhibited by MS could be attributed to the synergistic effects of some bioactive compounds in fresh red chili (such as luteolin and apigenin) and turmeric. Previous studies have indicated that red chili (*Capsicum annuum*) possesses the ability to reduce the production of NO and pro-inflammatory cytokines in macrophages stimulated with LPS [24], as well as in rats with carrageenan-induced paw edema [25]. Meanwhile, it was observed that the red curry pastes contained low quantities of each component. Consequently, the red curry paste demonstrated diminished molecular-level activity after the digestion process.

The mechanism underlying anti-inflammation in cells could be explained by the inhibition of NF-kB activation, which relates to the gene transcription factor iNOS and pro-inflammatory cytokines, by lowering intracellular accumulation of reactive oxygen species (ROS), allowing limitation of pro-inflammatory substance production at the gene level [16]. In an unexpected manner, our study investigated the potential of MS and MR to modulate the release of NO while having a minimal impact on the expression of iNOS. The observed phenomenon could potentially be attributed to the presence of bioactive compounds with antioxidant properties in the herbs. The majority of water-soluble bioactive compounds possess the capacity to scavenge free radicals in macrophages [26], consequently leading to the indirect inhibition of NO production without directly targeting the expression of iNOS.

Pathological changes occurring in the intestinal epithelium can be attributed to the presence of inflammation, resulting in a compromised mucosal layer barrier function [6, 7]. The present study presents the protective effects of a modified formulation of digested curry paste enriched with a four-fold concentration of turmeric on TEER of intestinal epithelial cells in the presence of activated macrophages. The findings of this investigation reveal that the application of the low concentration range of digested curry paste effectively prevented the TEER of intestinal epithelial cells from the deleterious impact induced by activated macrophages. The potential therapeutic effects of curcuminoid compounds obtained from the digestion of turmeric have been previously investigated in relation to the restoration of tight junction protein production. It is therefore suggested that the curcuminoids could

protect important tight junction proteins, including ZO-1 and occludin proteins, thereby preserving the normal barrier function of cells [27]. Moreover, it has been observed that the negative impacts induced by TNF-α and NO on basolateral intestinal epithelial cells may result in an augmented secretion of IL-8 from these cells [28]. IL-8 has been shown to have a role as a promoter of inflammatory processes, such as neutrophil infiltration in mucosal regions and inflamed wound sites, among other activities [9]. The regulation of IL-8 in intestinal cell monolayers has been found to be associated with TEER. It has been observed that as TEER decreases, there is a concomitant increase in IL-8 levels, indicating a possible role of IL-8 in modulating TEER [29]. The ultimate outcome was a decrease in the pathology of intestinal epithelial cells, which illustrates the concept of the *in vitro* anti-inflammatory mechanism in the intestinal epithelium protection by our modified Thai curry pastes. However, there is a limitation when using a higher concentration, which manifests as reduced protection offered to the intestinal epithelium. This limitation requires optimization for future investigations.

Furthermore, the transportation impact of curcumin through the intestinal monolayer was assessed in an *in vitro* system utilizing Caco-2/RAW264.7, in addition to biochemical studies. The findings indicated that curcumin within the gastrointestinal tract was localized exclusively on the surface of the intestinal epithelial cell layer facing the lumen (apical side), with no presence of curcumin on the basolateral side of the cell layer. The limited concentration of remaining curcumin in the *in vitro* system may be attributed to the significant dilution of the sample during the *in vitro* experiment. Ultimately, while being transported across the cell monolayer, the curcumin would get further diluted with the media in the basolateral compartment. In addition to the dilution effects, the study indicated that curcumin found in food items has limited water solubility and low bioavailability, due to its hydrophobic nature and poor physicochemical properties in the biological environment [30]. Thus, the movement of native curcumin from the apical side to the basolateral side is characterized by very poor permeability. Consistent with prior findings, the basolateral samples from the Caco-2/HT29-MTX cell model did not contain any detectable natural curcumin [31]. The basolateral side of a Caco-2 cell monolayer model showed a detection of just 4% of the natural curcumin, in comparison to the apical side [32]. In addition, a portion of the natural curcumin was taken up by the Caco-2 cells, as evidenced by the presence of curcumin in the cell homogenate. After being absorbed by the cells, curcumin undergoes metabolism, and its metabolites may be found in the basolateral fraction [32]. The study on the metabolism of curcumin in Caco-2 in the Caco-2 cell Millicell® system supported that curcumin is metabolized by Caco-2 cells after 3 h into reductive metabolites (e.g., hexahydrocurcumin and octahydro-curcumin) and subsequently conjugated with glucuronic acid and sulfate. Curcumin from the apical side disappeared after 6 h and appeared at very low concentrations on the basolateral side [33]. This finding suggests that curcumin metabolites, rather than curcumin itself, could be responsible for the observed anti-inflammatory activity in the basolateral chamber. Additionally, other bioactive components, such as polyphenols, could have contributed to the anti-inflammatory effects, as we could detect total phenolic compounds in the culture medium on the basolateral side. Hence, further research is necessary to discover these metabolites. Research has indicated that curcumin and other bioactive substances may contribute to the protection of cells by improving the processes that maintain the integrity of the intestinal epithelial barrier. This, in turn, helps avoid damage to cells caused by inflammation [34].

CONCLUSION

This study provides *in vitro* evidence that sheds light on the promising potential of southern Thai curcumin-rich curry pastes in the prevention and treatment of inflammatory gastrointestinal disorders. Anti-inflammatory properties are possessed by the bioactive components of the sour curry paste that contains turmeric. These components inhibit the synthesis of pro-inflammatory substances in macrophages and have the potential to protect intestinal epithelial cells from the harm caused by inflammation. These findings may provide scientific data for future studies and the development of novel interventions in the field of gastroenterology.

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