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Inhibition of *Escherichia coli* heat-labile enterotoxin-induced diarrhea by *Chaenomeles speciosa*

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is responsible for millions of deaths in developing countries. Heat-labile enterotoxin (LT), the virulence factor of ETEC, induces diarrhea by initially binding to the G_{M1} on the surface of intestinal epithelial cells and consequently leading to the massive loss of fluid and ions from cells. Fruit of *Chaenomeles* (FC), the dried fruit of *Chaenomeles speciosa*, has been used for diarrhea in China. However, the anti-diarrheal mechanism of FC is still unclear. In this study, we demonstrated that FC extract (FCE) inhibited the LT-induced diarrhea in mice by blocking the binding of the B subunit of LT (LTB) to G_{M1}. The ethyl acetate (EA) soluble fraction was the most active fraction of FC that significantly abolished the LTB and G_{M1} interaction. Furthermore, the oleanolic acid, ursolic acid, and betulinic acid from EA fraction, blocked the toxin binding effects, resulting in the suppression of LT-induced diarrhea. Moreover, by docking techniques, these compounds fitted LTB well via hydrogen bonds and hydrophobic contacts with amino acid residues of LTB. In conclusion, our findings suggested that oleanolic acid, ursolic acid, and betulinic acid were the active constituents from FC and might be considered as lead therapeutic agents in the treatment of LT-induced diarrhea.

Keywords: Enterotoxigenic *Escherichia coli*-induced diarrhea; Fruit of *Chaenomeles*; Heat-labile enterotoxin; Oleanolic acid; Ursolic acid; Betulinic acid

1. Introduction

Diarrheal disease remains a leading global health problem. It has been estimated that 2 billion to 4 billion episodes of infectious diarrhea and 3 million to 5 million deaths from diarrhea occur annually in developing countries (Sanchez and Holmgren, 2005). Enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of diarrhea in human beings. It accounts for more than 200 million cases of diarrhea and approximately 380,000 deaths annually among children under 5 years of age (WHO, 2002). ETEC is also the most common pathogen of traveler's diarrhea that affects 10 million travelers to developing countries (Aranda-Michel and Giannella, 1999).

Heat-labile enterotoxin (LT) is the major virulent factor of ETEC (Holmgren and Svennerholm, 1992). LT comprises of one A subunit and five identical B subunits (Merritt and Hol, 1995). The mechanism of diarrhea induced by LT is initiated by the binding of B subunits (LTB) to the ganglioside G_{M1} [Galβ1-3GalNAcβ1-4 (Neu5Acα2-3)Gal-β1-4Glc-Ceramide], on the surface of intestinal epithelial cells (Spangler, 1992; Pickens et al., 2002). Binding of LTB to G_{M1} induces a conformational change in the toxin molecule, followed by the translocation of A subunit (LTA) into the cells. Inside the intestinal cells, LTA

Abbreviations: CT, cholera toxin; EA, ethyl acetate; ETEC, enterotoxigenic *Escherichia coli*; FCE, fruit of *Chaenomeles* extract; G_{M1}-ELISA, G_{M1}-enzyme-linked immunosorbent assay; LT, heat-labile enterotoxin

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catalyzes the ADP-ribosylation of the stimulatory GTP-binding protein, resulting in increased intracellular levels of cyclic AMP. Elevated levels of cyclic AMP in the cells cause massive loss of fluid and ions from the cell, then leading to the symptom of diarrhea (Spangler, 1992). Since the interaction between toxin and receptor is the first step of LT-induced diarrhea, the binding of LTB to G_{M1} is therefore an attractive target for developing drugs or prophylactics for the treatment and prevention of LT-induced diarrhea (Merritt et al., 1997; Minke et al., 1999; Pickens et al., 2002; Mitchell et al., 2004).

In our previous study, we have already set up an *in vitro* model to evaluate the binding ability of LTB to G_{M1}. There are many Chinese medicinal plants, including the cortex of *Cinnamomum*, the rhizome of *Coptidis* and the fruit of *Chaenomeles*, ethnomedically used for the treatment of cholera-like diarrhea. Therefore, we selected a number of Chinese medicinal plants on the basis of ethnomedical use for anti-diarrhea and evaluated their effects on the LTB and G_{M1} interaction. Our data showed that *Galla chinensis* is capable of inhibiting the binding of LTB to G_{M1}, resulting in the suppression of LT-induced diarrhea in mice (Chen et al., 2006). Recently, we found another Chinese medicinal herb, fruit of *Chaenomeles*, was also effective in blocking the binding of LTB to G_{M1}. Fruit of *Chaenomeles* (Mu-Gua) is the dried fruit of several *Chaenomeles* species, including *Chaenomeles speciosa* (Sweet) Nakai. Fruit of *Chaenomeles* has been used in traditional Chinese medicine and other oriental medicine systems for years. It has long been used for healing gastrointestinal disorders, such as gastroenteritis, arthritis, and diarrhea in China (ChPC, 2005). Fruit of *Chaenomeles* contains various components, such as oleanolic acid, ursolic acid, betulinic acid, stearic acid, β-sitosterol, palmitic acid, ursolic acid-3-*O*-behenate, 3-acetyl ursolic acid, 3-acetyl pomolic acid, and daucosterol (Chen and Wei, 2003; Li and He, 2005). Glucosides of *Chaenomeles speciosa* possesses anti-inflammatory and immunoregulatory actions and has a therapeutic effect on collagen-induced arthritis *in vivo* (Chen and Wei, 2003; Zhang et al., 2004). Fruit of *Chaenomeles* has also been applied in the treatment of diarrhea in China; however, the anti-diarrheal effect of fruit of *Chaenomeles* was still unclear. Therefore, the aim of this study was to examine the anti-LT-induced diarrheal efficacy of fruit of *Chaenomeles* by G_{M1}-enzyme-linked immunosorbent assay (ELISA) and patent gut assay. The active components of fruit of *Chaenomeles* responsible for the anti-diarrheal activity were further analyzed. Since the prediction of ligand binding sites is an essential part of new drug discovery, we further calculated the binding sites between LTB and active components of fruit of *Chaenomeles* for lead compounds identification and optimization in the future.

2. Materials and methods

2.1. Plant materials, extraction and fractionation of fruit of Chaenomeles, and compounds

The crude extract powder of fruit of *Chaenomeles* (*Chaenomeles speciosa* (Sweet) Nakai) (Voucher No. 5500) was purchased from Kaiser Pharmaceutical Co., Ltd. (Taiwan). The HPLC profiling of fruit of *Chaenomeles* is provided in Supplementary Data. The crude extract powder (100 g) was re-extracted with methanol by percolation until complete exhaustion. The methanol extract of *Chaenomeles* was concentrated under reduced pressure at a temperature less than 40 °C, divided into small aliquots, and kept at −30 °C until further use. The fruit of *Chaenomeles* extract (FCE) was suspended in distilled water and then partitioned with four different solvents (*n*-hexane, chloroform, ethyl acetate, *n*-butanol) to yield *n*-hexane, chloroform, ethyl acetate (EA), *n*-butanol, and aqueous fractions, respectively. Each fraction was concentrated under reduced pressure at a temperature less than 40 °C, and the solid mass was then dissolved in methanol, divided into small aliquots, and kept at −30 °C until use. Oleanolic acid, ursolic acid, and betulinic acid were purchased from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide at 100 mM.

2.2. Expression and purification of Escherichia coli LT and LTB

Recombinant LT and LTB were expressed in *Escherichia coli* BL21(DE3)pLysS strain and purified by affinity chromatography as described previously (Chen et al., 2006). Briefly, cells were grown in 100 ml Luria–Bertani broth agitated at 37 °C until OD_{600} reached 0.6. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM and the cells were collected 3 h after induction. The cell pellet was resuspended in 4 ml 1× TEAN buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 200 mM NaCl, 3 mM NaN_3), lysed by sonication, and centrifuged at 15,000 × *g* for 20 min at 4 °C. The supernatant was collected and mixed with D-galactose resin (Pierce, Rockford, IL, USA), and the recombinant LT and LTB were then eluted by 1× TEAN buffer containing 1 M galactose. Proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and quantified with a Bradford assay (Bio-Rad, Hercules, CA, USA).

2.3. Patent mouse gut assay

Female BALB/c mice (8 weeks old, 20 ± 1 g weight) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Mouse experiments were conducted under ethics approval from the China Medical University Animal Ethics Committee.

In vivo LT-induced diarrheal ability was determined by the patent mouse gut assay as described previously (Baselski et al., 1997; Chen et al., 2006). Briefly, five mice per group were starved with water only for 16 h. Each mouse was inoculated intragastrically with 0.5 ml of 10 μg LT alone or in conjunction with various amounts of FCE. Six hours latter, the mice were sacrificed. The entire intestine from duodenum to rectum was carefully removed to retain any accumulated fluid, and the residual mesentery was removed prior to weighing. The carcass was weighed separately. LT-induced diarrheal ability was presented as a gut/carcass weight ratio as followed. The IC_{50} value of compound was determined as the quantity of compound required to

inhibit the LT-induced gut/carcass ratio at 50%

$$\text{gut/carcass ratio} = \frac{\text{gut weight (g)}}{\text{carcass weight (g)}}$$

2.4. Biotinylation of LTB

LTB was mixed with Sulfo-NHS-LS-biotin (Pierce, Rockford, IL, USA) in a ratio of 1:10. After a 2 h-incubation on ice, the unincorporated biotin was removed by centricon-10 (Millipore, Bedford, MA, USA), and the biotinylated LTB was stored at 4 °C until further analysis. Sulfo-NHS-LS-biotin should be prepared freshly by dissolving in water.

2.5. Competitive G_{M1}-ELISA

Biotinylated LTB (16 ng) was mixed with various amounts of compound and incubated at 4 °C for 3 h with shaking. Microtiter plates (MaxiSorp Nunc-Immum™ plates, Nunc, Denmark) were coated at 4 °C overnight with 100 μl of 2 ng/μl G_{M1} (Sigma, St. Louis, MO, USA), which was diluted in phosphate-buffered saline (PBS) (137 mM NaCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, pH 7.2). The wells were washed with 200 μl washing buffer (0.5% Tween 20 in PBS), blocked with 200 μl blocking buffer (1% bovine serum albumin (BSA) in PBS) at 37 °C for 1 h, and then incubated with 100 μl of biotinylated LTB/compound mixture at 37 °C for 1 h. After three washes with washing buffer, 50 μl of diluted peroxidase-conjugated avidin (Pierce, Rockford, IL, USA) was added to each well and incubated at 37 °C for 1 h. Following three washes, 50 μl of chromogenic substrate, 2,2′-azinobis(3-ethylbenzthiazoline-sulfonic acid) (Sigma, St. Louis, MO, USA), was added to each well and incubated at 37 °C for 15 min. The absorbance was read at 405 nm in an ELISA plate reader. The inhibitory ability (%) was calculated by [1-(OD value of mixture containing LTB and compound/OD value of mixture containing LTB only)] × 100.

2.6. Thin layer chromatography (TLC) analysis

Samples were submitted to the characterization by TLC (silica gel 60 F254, Merck; development twice with the same mobile phase: *n*-hexane/ethyl acetate/glacial acetic acid, 7:3:0.3). To confirm the presence of triterpenes, chromatograms were sprayed with 5% vanillin/H_2SO_4 solution and evaluated under visible light.

2.7. Docking technology

The MEDock (Maximum Entropy based Docking) web server (http://medock.csie.ntu.edu.tw/) is used for the prediction of ligand binding sites (Chang et al., 2005; Van Dijk et al., 2005). The input file is in the PDBQ format, which is an extension of the PDB format. The PDBQ format for ligands (triterpenes) has been generated by Dundee's PRODRG server (http://davapc1.bioch.dundee.ac.uk/programs/prodrg/) (Schuttelkopf and van Aalten, 2004). The PDB file (PDB code 1LTS) of LT was taken from the Protein Data Bank (http://www.rcsb.org/pdb/) (Sixma et al., 1993). The PDBQ file for LT has been derived from the PDB2PQR server (http://agave.wustl.edu/pdb2pqr/) (Dolinsky et al., 2004).

2.8. Statistical analysis

Data were presented as mean ± standard error. Student's *t*-test was used for a comparison between two experiments. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. FCE suppressed the LT-induced fluid accumulation in mice

Since one of the biological activities of LT is the induction of fluid accumulation in the intestine, we analyzed the anti-diarrheal effect of FCE by patent mouse gut assay. FCE significantly suppressed the LT-induced fluid accumulation with a concentration-dependent decrease in the gut/carcass ratio (Fig. 1). These results indicated that FCE was able to inhibit LT-induced diarrhea, with the IC_{50} value of 2.4 ± 0.6 mg/ml.

3.2. FCE suppressed the LT-induced fluid accumulation by blocking the binding of LTB to G_{M1}

The inhibitory ability of FCE on the binding of LTB to G_{M1} was evaluated by competitive G_{M1}-ELISA. Various amounts of FCE were mixed with 16 ng of biotin-labeled LTB, incubated at 4 °C for 3 h, and added to G_{M1}-coated wells. FCE significantly inhibited the binding of LTB to G_{M1} (Fig. 2). The inhibitory effect of FCE displayed a concentration-dependent manner, with the IC_{50} value of 193.1 ± 15.2 μg/ml. Therefore, these data indicated that FCE blocked the binding of LTB to G_{M1}, resulting in the suppression of LT-induced diarrhea.

3.3. EA and n-butanol fractions of FCE blocked the binding of LTB to G_{M1}

We further analyzed the effects of various FCE fractions on the binding of LTB to G_{M1} by G_{M1}-ELISA. As shown in Fig. 3,

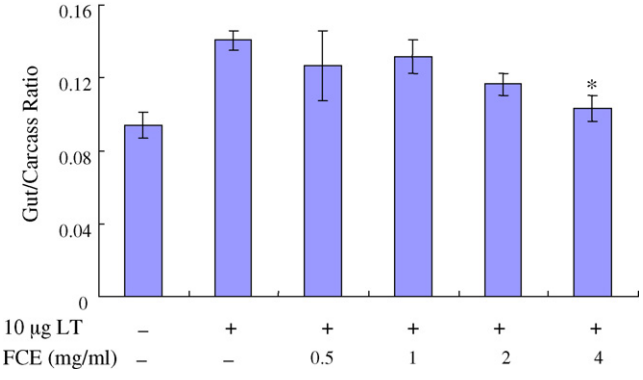

Fig. 1. Anti-diarrheal effect of FCE by patent mouse gut assay. Values are mean ± standard error of triplicate assays. $^{*}p < 0.05$, compared with LT treatment.

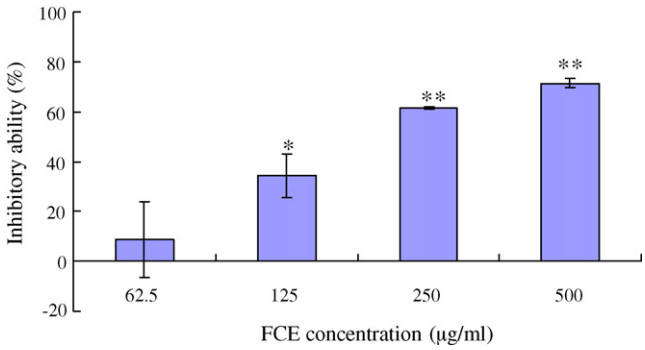

Fig. 2. Inhibitory ability of FCE on the binding of LTB to G_{M1} by competitive G_{M1}-ELISA. Values are mean ± standard error of four independent assays. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with LTB.

EA and *n*-butanol fractions significantly inhibited the binding of LTB to G_{M1} in a concentration-dependent manner, with the IC_{50} value of 114.4 ± 5.4 and 118.6 ± 8.9 μg/ml, respectively.

3.4. Triterpenes from FCE exhibited the anti-LT-induced diarrhea abilities by blocking the binding of LTB to G_{M1}

Because fruit of *Chaenomeles* contains various triterpenes (Chen and Wei, 2003; Li and He, 2005), we determined the presence of triterpenes in the EA fraction of FCE by TLC. As shown in Fig. 4, the major components of EA fraction were triterpene derivatives. Several oleanane, ursane, and lupine groups of triterpenes have been isolated from the fruit of *Chaenomeles* and the representatives of these triterpenes are oleanolic acid, ursolic acid, and betulinic acid. Therefore, we analyzed three representative triterpene components for their anti-diarrheal effects by competitive G_{M1}-ELISA and patent mouse gut assay. Oleanolic acid, ursolic acid, and betulinic acid significantly blocked the binding of LTB to G_{M1}, with the IC_{50} value of 202.8 ± 47.8, 493.6 ± 100.0, and 480.5 ± 56.9 μM, respectively (Fig. 5). All of these triterpene components also significantly suppressed the LT-induced fluid accumulation at 4 mM (Fig. 6). Therefore, these findings indicated that the triterpenes were the likely constituents

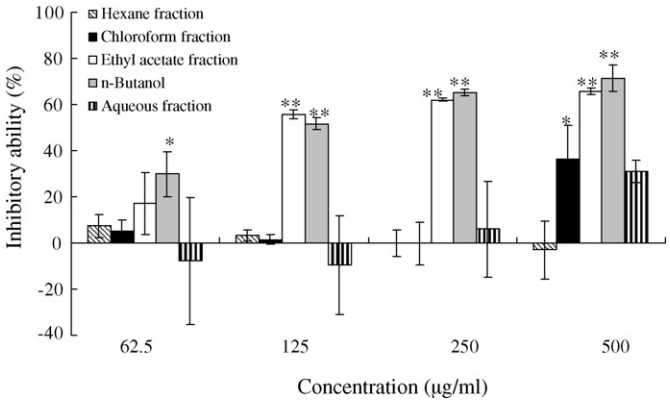

Fig. 3. Inhibitory abilities of FCE fractions on the binding of LTB to G_{M1} by competitive G_{M1}-ELISA. Values are mean ± standard error of four independent assays. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with LTB.

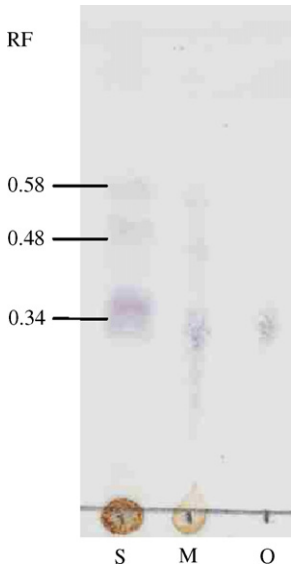

Fig. 4. Identification of triterpenes in the EA and *n*-butanol fractions of FCE by TLC. S represents EA fraction of FCE, M represents the mixture containing EA fraction of fruit of *Chaenomeles* and oleanolic acid, and O represents oleanolic acid.

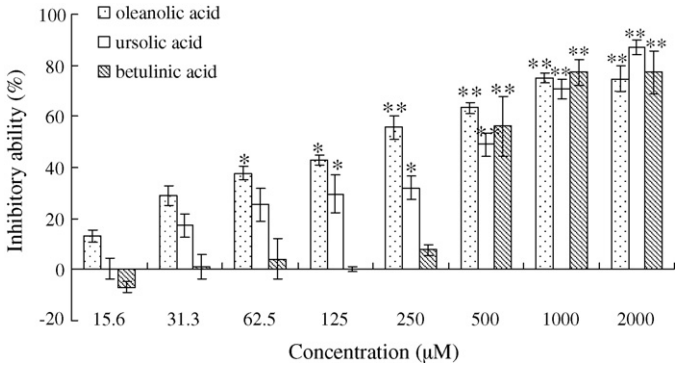

Fig. 5. Inhibitory abilities of triterpenes on the binding of LTB to G_{M1} by competitive G_{M1}-ELISA. Values are mean ± standard error of four independent assays. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with LTB.

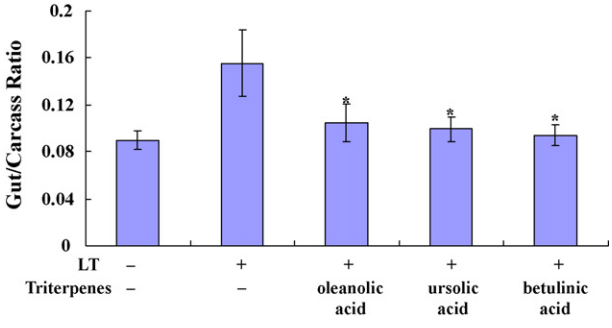

Fig. 6. Anti-diarrheal effects of triterpenes by patent mouse gut assay. Values are mean ± standard error of triplicate assays. $^{*}p < 0.05$, compared with LT treatment.

from fruit of *Chaenomeles* for the suppression of LT-induced diarrhea.

3.5. Docking analysis of the interaction between triterpenes and LT

We further interpreted the binding sites of triterpenes in LT by docking technology. Oleanolic acid, ursolic acid, and betulinic acid were capable of docking into the B subunit of LT (Fig. 7). These compounds fitted LTB very well, with the predicted binding energy score of −11.76, −12.07, and −10.99 kcal/mol, respectively. Critical hydrogen bonds with the Glu^{51}, His^{57}, and Gln^{61} residues of LTB were found. Moreover, compounds exhibited hydrophobic contacts with the Gly^{33}, Trp^{88}, and Lys^{91} residues as well as with the peptide backbone of LTB. In addition to the aforementioned interaction with LTB, compounds also exhibited unique hydrophobic interactions with LTB. Oleanolic acid exhibited additional hydrophobic contact with Lys^{34} residue of LTB. Hydrophobic interactions with Tyr^{12} and Arg^{13} residues were found in ursolic acid. Betulinic acid exhibited additional hydrophobic contacts with Gln^{56} residues of LTB.

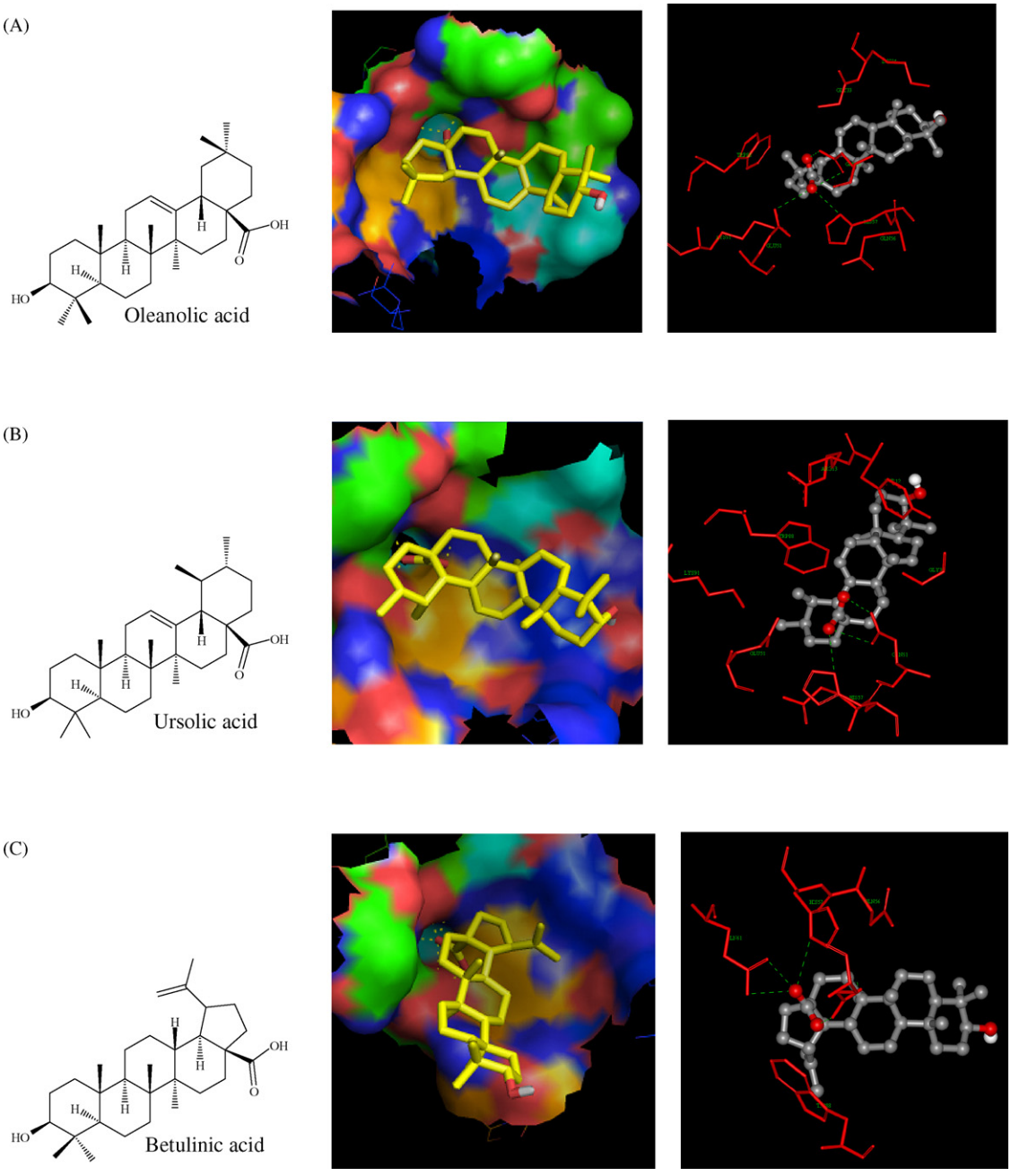

Fig. 7. Docking structure between LT and triterpenes. Chemical structures of oleanolic acid (A), ursolic acid (B), and betulinic acid (C) are shown on the left panel. Surface representations of LTB complexed with triterpenes are shown on the middle panel. Close up views of LTB complexed with triterpenes are shown on the right panel. Triterpenes are represented by stick style. Side chains of selected residues are represented by red wire style. Hydrogen bonds between LTB and triterpenes are represented by green dash line.

4. Discussion

In this study, we demonstrated that FCE suppressed diarrheal symptoms induced by LT in mice. Various reagents, such as castor oil, arachidonic acid, prostaglandin E2, magnesium sulphate and enterotoxins, have been used for the establishment of diarrheal models in animals. The mechanisms of diarrhea induced by various inducers are totally different. For examples, castor oil is an effective laxative by decreasing fluid absorption, increasing secretion in the small intestine, and affecting smooth muscle contractility in the intestine (Pierce et al., 1971; Chitme et al., 2004). Arachidonic acid and its metabolite, prostaglandin E2, inhibit sodium and chloride absorption from mucosal surface and cause stimulation of motility and conversion of small intestinal mucosa from absorption to secretion of water and electrolytes (Declusin et al., 1974; Jaffe, 1979). Magnesium sulphate induces diarrhea by preventing the reabsorption of water ions, leading to increment of the volume of the intestinal content (Zavala et al., 1998).

In ancient Chinese medicine, fruit of *Chaenomeles* is used for the treatment of cholera-like diarrhea. Our findings revealed that extracts from the fruit of *Chaenomeles* indeed inhibited LT-induced diarrhea in mice via the blockade of the LTB and G_{M1} interaction. The clinical symptoms of ETEC infection might range from the mild diarrhea to the severe cholera-like syndrome (Qadri et al., 2005). ETEC strains generally colonize on the surface of the small bowel mucosa and cause diarrhea through the action of LT. Therefore, killing *Escherichia coli* or blocking LT action might be targets for the development of drugs for treating cholera-like diarrhea. The antibacterial activities of FCE and triterpenes against *Escherichia coli* have been evaluated by MTT colorimetric assay. Neither the FCE nor the triterpenes exhibited antibacterial activities at 4 mg/ml or 2 mM, respectively (data not shown). Therefore, these results indicated that FCE and triterpenes inhibited LT-induced diarrhea not via killing *Escherichia coli* but through blocking the binding of LTB to G_{M1} receptor.

Traditional medicines have been used for people's health care in many developing countries (Kim, 2005). Since there are approximately 500,000 plant species occurring worldwide, of which only 1% has been phytochemically investigated, there is great potential for discovering novel bioactive compounds (Groombridge and Jenkins, 2002). The phytochemicals derived from medicinal plants have proven to be an abundant source of specific biologically active compounds, many of which have been the basis for the development of new lead chemicals for pharmaceuticals (van Der et al., 2004). For examples, vinblastine and vincristine are natural products, belonging to the group of terpenoid–indole alkaloids. They are isolated from the pantropical plant *Catharanthus roseus* (Madagascar periwinkle, formerly known as *Vinca rosea*). In the previous study, we identified that *Galla chinensis* and gallic acid exhibited anti-diarrheal activities via the abolishment of LTB and G_{M1} interaction. Gallic acid significantly blocked the binding of LTB to G_{M1} and inhibited LT-induced diarrhea, with the IC_{50} value of 10.9 ± 0.3 mM and 25.4 ± 11.6 mM, respectively (Chen et al., 2006). However, the IC_{50} values of triterpenes from the fruit of *Chaenomeles* for *in vitro* and *in vivo* assays were less than 0.5 and 4 mM, respectively. Therefore, these findings suggested that triterpenes compounds (oleanolic acid, ursolic acid, and betulinic acid) might be considered as lead therapeutic agents in the treatment of LT-induced diarrhea.

Numerous studies have validated the traditional use of anti-diarrheal medicinal plants by investigating the specific activity of extracts of such plants, which have antispasmodic effects, delay intestinal transit, suppress gut motility, stimulate water adsorption, or reduce electrolyte secretion (Palombo, 2006). Some of phytochemicals are present in the active extracts and exhibit anti-diarrheal abilities. For examples, tannins and flavonoids are thought to be responsible for anti-diarrheal activity through increasing colonic water and electrolyte reabsorption, or directly inhibiting functions of enterotoxins (Oi et al., 2002; Vidari et al., 2003). Simple phenolics and proanthocyanidin have been shown to inhibit Cl^- secretion (Gabriel et al., 1999; Ogata and Shibata, 2004). Alkaloids, such as piperine, might act by inhibiting intestinal motility (Bajad et al., 2001). In addition to these compounds, there are few studies indicated that triterpenes exhibited anti-diarrheal effects. Oleanolic acid, ursolic acid, and betulinic acid are ubiquitous pentacyclic triterpenes in plant kingdom, and lots of biological, pharmacological, and toxicity effects have been demonstrated. For examples, these compounds exhibit anti-inflammatory, anti-microbial, anti-oxidant, anti-tumor, and hepatoprotective effects. Moreover, their gastroprotective and anti-ulcer effects have been reported (Ovesna et al., 2004; Liu, 2005; Yogeeswari and Sriram, 2005). In this study, we demonstrated that oleanolic acid, ursolic acid, and betulinic acid were capable of suppressing the LT-induced diarrhea at 4 mM, which is less than the LD_{50} in mice (Singh et al., 1992). By docking techniques, we also predicted that carboxylic groups of these compounds might be the major functional group for binding. Therefore, our findings suggested that oleanolic acid, ursolic acid, and betulinic acid might be considered as lead therapeutic agents in the treatment of LT-induced diarrhea.

LT and cholera toxin (CT) are very similar in several ways. For examples, both are the members of AB5 toxin family. They consist of one A subunit with ADP-ribosylation activity and five B subunits for receptor binding. They not only share a 80% sequence homology but also share the same receptor-ganglioside G_{M1}. Additionally, they cause cholera-like diarrhea in clinical (Spangler, 1992). Docking analysis of B subunits with G_{M1} shows the perfect match in the binding sites (Supplementary Data). Therefore, it is reasonable to expect that oleanolic acid, ursolic acid, and betulinic acid were capable of blocking the binding of CT to G_{M1}. Additionally, because the binding of toxin to G_{M1} is the first step for the translocation of A subunit into cells, blocking the toxin and G_{M1} interaction would abort the subsequent events, such as the ADP-ribosylation of G protein and the elevation of cyclic AMP.

5. Conclusions

We demonstrated that FCE suppressed the LT-induced fluid accumulation by blocking the binding of LTB to G_{M1}. Oleanolic acid, ursolic acid, and betulinic acid abolished the interaction of LTB to G_{M1}, resulting in the suppression of LT-induced diarrhea.

Therefore, these data suggested that oleanolic acid, ursolic acid, and betulinic acid from fruit of *Chaenomeles* might be potent inhibitors for the treatment of LT-induced diarrhea.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jep.2007.05.031.

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