The Promotive Effect of the Active Ingredients of Atractylodes macrocephala on Intestinal Epithelial Repair Through Activating Ca\(^{2+}\) Pathway

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Abstract

Atractylodes macrocephala (AM) is a famous traditional Chinese medicine for intestinal epithelial restitution through activating Ca\(^{2+}\) channels. However, the roles of specific AM compositions in intestinal epithelial restitution are sparse. Therefore, this study aimed to compare the concrete effects of the 4 active ingredients (atractylon, \(\beta\)-eudesmol, atractylenolide II, atractylenolide III) of AM and their combination on intestinal epithelial repair and the Ca\(^{2+}\) pathway in intestinal epithelial cell (IEC-6) cells. First, the best combination of the 4 ingredients with an optimal mixing ratio of atractylon: \(\beta\)-eudesmol: atractylenolide II: atractylenolide III = 1:2:2:2 was demonstrated by a 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide orthogonal experiment. Subsequently, enzyme-linked immunosorbent assay was used to measure anti-inflammatory cytokine levels, the migratory ability was evaluated by cell scratch experiments, cell cycle analysis and [Ca\(^{2+}\)]\(_{cyt}\) concentration in cells were detected by flow cytometry, and the expression of the Ca\(^{2+}\) pathway-related genes was detected by immunofluorescence staining, quantitative polymerase chain reaction and whole blood assays. Our result showed that atractylon, \(\beta\)-Eudesmol, atractylenolide II, and atractylenolide III showed different abilities to promote the IEC-6 cells proliferation, migration, and the expression of anti-inflammatory cytokines interleukin (II)-2, II-10, and ornithine decarboxylase, as well as the intracellular [Ca\(^{2+}\)]\(_{cyt}\) concentration through stromal interaction molecule 1 transposition to activate Ca\(^{2+}\) pathway. Thereinto, atractylenolide III was the main active ingredient of AM for pro-proliferation and anti-inflammation, and the combination of 4 AM ingredients performed better beneficial effects on IEC-6 cells. Therefore, our study suggested that atractylenolide III was the active ingredient of AM for intestinal epithelial repair through activating the Ca\(^{2+}\) pathway, and the 4 ingredients of AM have a synergy in intestinal epithelial repair.

Keywords

Atractylodes macrocephala, Ca\(^{2+}\) pathway, IEC-6, intestinal epithelial repair

Introduction

The gastrointestinal (GI) tract acts as one of the greatest barriers between the external and internal environments and the integrity of this barrier plays a pivotal role in maintaining homeostasis.\(^1\) Intestinal mucosal is not only involved in the digestion and absorption of nutrients, mostly, provides a selectively permeable barrier against endogenous and exogenous antigens.\(^4,5\) Intestinal barrier defects have been shown in a broad range of diseases, containing inflammatory bowel disease (IBD), colon carcinoma, chronic liver disease, type 1 diabetes, obesity, and so on.\(^6\) Remarkably the basic structure of barriers between the environment and the internal host milieu is formed by mucosal epithelial cells.\(^7,8\) During the early epithelial restitution of the GI mucosa, the functions of intestinal epithelial cells (IECs) including migration and metabolism are important.\(^6,9-14\) The cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{cyt}\)) plays a significant role in the regulation of epithelial restitution after injury.\(^13,15,16\) Experiments in vitro and in vivo have shown that [Ca\(^{2+}\)]\(_{cyt}\) promotes intestinal epithelial restitution after wounding.\(^\)\(^13,15,16\) Canonical transient receptor potential 1 (TRPC1) are store-transported Ca\(^{2+}\) channels that control the balance of [Ca\(^{2+}\)]\(_{cyt}\) to influence epithelial restitution.\(^15\) Stromal interaction molecule

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1 (STIM1) and stromal interaction molecule 2 (STIM2) are the sensors of Ca\(^{2+}\) within the store, acting as essential parts in regulating TRPC1-mediated Ca\(^{2+}\) influx after store depletion.\(^{19}\)

*Atractylodes macrocephala (AM)* is an important traditional Chinese medicine which is widely used to treat chronic intestinal disease.\(^{20}\) Recently, there has been an increasing interest in exploring the pharmacological roles of this drug. Some studies have proved that AM promoted intestinal epithelial restitution through the Ca\(^{2+}\) channels.\(^{21,22}\) AM is comprised of many substances, however, the studies in the role of specific AM composition are sparse.

IECs play a key role in the initiation and perpetuation of intestinal inflammation. And the intestinal epithelial (enterocyte) cell line IEC-6 is often used as an intestinal inflammation model system in vitro.\(^{23}\) Previous studies showed that atracylone, β-eudesmol, atracylenolide II, and atracylenolide III were the main compositions of AM,\(^{24-28}\) however, their impact on intestinal repair has been less studied. So, in this study, we aimed to compare the concrete effect of the 4 AM active ingredient atracylone, β-eudesmol, atracylenolide II, and atracylenolide III was as well as the combination on the intestinal epithelial repair through the Ca\(^{2+}\) pathway.

**Result**

The Pro-Proliferation of 4 AM Effective Ingredients for IEC-6 Cells

As shown in Figure 1, atracylone (10-160 μM), β-eudesmol (10-40 μM), atracylenolide II (10-40 μM), and atracylenolide III (10-20 μM) significantly increased the survival rate of IEC-6 cells after 24 h co-culture, while atracylone (10-160 μM), β-eudesmol (10-40 μM), atracylenolide II (10-20 μM) and atracylenolide III (10-20 μM) also showed a significant increase after 48 h co-culture. Notably, the excess β-eudesmol (≥ 80 μM), atracylenolide II (≥ 160 μM), and atracylenolide III (≥ 40 μM) were cytotoxic to normal cells.

![Figure 1](image-url)

*Figure 1.* The pro-proliferation of the 4 AM effective ingredients for IEC-6 cells. IEC-6 cells were incubated with atracylone, β-eudesmol, atracylenolide II, and atracylenolide III for 24 h or 48 h, respectively. After incubation, the survival rate was determined by MTT assay. \(n=6\).
* denote \(P<.05\) versus the control group (0 μM of active ingredients of AM).

Abbreviations: AM, Atractylodes macrocephala; IEC-6, intestinal epithelial cell 6; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide.
Analysis of the Optimal Mixing Ratio to Promote the IEC-6 Cells Proliferation

According to the results of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (Figure 2), the concentrations from 10 to 40 μM were employed for the L9 (3⁴) orthogonal test to choose the optimal mixing ratio of the 4 active ingredients. The result of the L9 (3⁴) orthogonal test showed that the best combination to promote the IEC-6 cells proliferation was consisted by 10 μM atractylon, 20 μM β-eudesmol, 20 μM atractylenolide II, and 20 μM atractylenolid III. Thus, the optimal mixing ratio was atractylon: β-eudesmol: atractylenolide II: atractylenolid III = 1:2:2:2.

The Anti-Inflammatory Effect of 4 AM Effective Ingredients and Their Combination for IEC-6 Cells

The results of enzyme-linked immunosorbent (ELISA) for anti-inflammatory cytokines interleukin (IL)-2, IL-10, and ornithine decarboxylase (ODC), as shown in Figure 3, atractylon could only increase the ODC expression, and β-eudesmol only increased the IL-2 expression. While, both atractylenolide III and the combination with 1:2:2:2 mixing ratio significantly increased the expressions of anti-inflammatory cytokines IL-2, IL-10, and ODC. Moreover, the combination with 1:2:2:2 mixing ratio had a higher expression of IL-10 and ODC compared to atractylenolide III.

The Pro-Cell Motility and Cell Cycle of 4 AM Effective Ingredients and Their Combination for IEC-6 Cells

The results of the wound healing assay showed that the cell motility of IEC-6 cells was promoted by atractylon, β-eudesmol, Atractylenolide II, Atractylenolide III, and their combination. Thereinto, atractylenolide III performed a better pro-motility on ICE-6 cells in the 4 ingredients, while, the promotive effect of their combination was better than all the ingredients (Figure 4A). The results of the cell cycles as shown in Figure 4B, the 4 ingredients of AM and their combination could significantly increase the cells %age of G1 phase, while atractylon, atractylenolide III, and their combination decreased the cells %age of S phase in IEC-6 cells. Of them, the pro-cell cycle progression effects of atractylenolide III and atractylon were more obvious than the other 2 ingredients, and the pro-cell cycle progression effects of their combination were the most prominent in all groups. Those suggested that atractylon, atractylenolide III, and their combination promoted the proliferation of IEC-6 cells through advancing the cell cycle progression.

The Effects on STIM1 and STIM2 Expression in IEC-6 Cells

The result in Figure 5A showed that atractylon, β-eudesmol, atractylenolide II, atractylenolide III, and the combination all promoted the translocation of STIM1 to the plasma membrane.

Figure 2. Analysis for the optimal mixing ratio to promote the IEC-6 cells proliferation. L9 (3⁴) orthogonal test was used for grouping atractylon (0-40 μM), β-eudesmol (0-40 μM), atractylenolide II (0-40 μM), and atractylenolide III (0-40 μM). After 24 h incubation, the survival rate was determined by MTT assay. The result showed that the best combination was 10 μM atractylon, 20 μM β-eudesmol, 20 μM atractylenolide II, and 20 μM atractylenolid III, thus, the optimal mixing ratio was atractylon: β-eudesmol : atractylenolide II: atractylenolide III = 1:2:2:2. n = 6.

*, **, *** denote P < .05, P < .01, and P < .001 versus the group without any active ingredients of AM (0 μM).

Abbreviations: AM, Atractylodes macrocephala; IEC-6, intestinal epithelial cell 6; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
and the effect of atracylenolide III on the translocation of STIM1 was better than the other 3 ingredients, while the combination had a better effect than all ingredients. The results of quantitative polymerase chain reaction (q-PCR) and whole blood (WB) showed that atracylon, β-eudesmol, atracylenolide II, atracylenolide III, and the combination all had a significant impact to up-regulate STIM1 expression and down-regulate STIM2 expression. Similarly, the effect of atracylenolide III on STIM1 and STIM2 expressions was more obviously than the other 3 ingredients, and the combination had the largest effect in all groups (Figure 5B).

The Effects of 4 AM Effective Ingredients and Their Combination on Ca^{2+} Pathways in IEC-6 Cells

As shown in Figure 6A, atracylon, atracylenolide III, and the combination significantly increased the [Ca^{2+}]_{cyt} concentration of IEC-6 cells, and the effect of atracylenolide III on [Ca^{2+}]_{cyt} concentration of IEC-6 cells was better than atracylon, while the [Ca^{2+}]_{cyt} cell concentration of the combination was highest in all the groups. However, there was no difference in the [Ca^{2+}]_{cyt} concentration after β-eudesmol and atracylenolide II treatments. As shown in Figure 6B, atracylon and
atractylenolide II only significantly increased the expression of TRPC1 and RhoA protein, and β-eudesmol and atractylenolide III significantly enhanced the expression of TRPC1, PLC-γ1, and RhoA proteins, while atractylenolide III demonstrated a higher protein expression level than β-eudesmol. Moreover, the combination exhibited the strongest promoting effect on TRPC1, PLC-γ1, and RhoA proteins.

Discussion

AMI, as a famous Chinese medicine used to treat chronic intestinal disease, has been known to repair intestinal epithelium through the Ca2+ channels.21,22 However, there were fewer studies to elucidate the specific roles of concrete ingredients involved in this process. In this study, we investigated the role of the 4 ingredients atractylon, β-Eudesmol, atractylenolide II, and atractylenolide III of AM and their best combination on the intestinal epithelium repairment. Based on the results, we found that the 4 ingredients of AM could promote the proliferation of IEC-6 cells, but the excess β-eudesmol (≥80 μM), atractylenolide II (≥160 μM), and atractylenolide III (≥40 μM) had cytotoxicity to normal cells. Furthermore, we found that the 4 ingredients all had anti-inflammatory, pro-cell motility, and pro-cell cycle effects through activating Ca2+ pathways, while atractylenolide III exerted the most prominent effect in them. Additionally, we confirmed the best combination of the 4 AM ingredients to promote the proliferation of IEC-6 cells, which was formed by a 1:2:2:2 mixing ratio of the 4 AM ingredients, and the promotive effect of the 4 ingredients combination on proliferation, was better than they used separately.

The GI tract is the most susceptible target to inflammatory responses,29 and IBD are more likely to cause intestinal
Thus, the anti-inflammatory activity of intestinal epithelium is particularly important to intestinal epithelial repair. It has been reported that atractylenolide I to III had an anti-inflammatory activity for the GI tract. ODC, as a critical regulator of M1 macrophage activation, could inhibit M1 macrophage activation, and IL-2 and IL-10 are anti-

**Figure 5.** The effects of 4 AM effective ingredients and their combination on STIM1 and STIM2 expression. IEC-6 cells were incubated with ingredients (total 20 μM per group) or not for 24 h. After incubation, IEC-6 cells were determined by immunofluorescence staining WB and q-PCR, n = 3. (A) The immunofluorescence staining results of STIM1. (B) The WB results of STIM1 and STIM2 protein expression. (C) The q-PCR results of STIM1 and STIM2 mRNA expression. *, **, *** denoted $P < .05$, $P < .01$, and $P < .001$ versus the control group. #, ##, ### denote $P < .05$, $P < .01$, and $P < .001$ versus combination group. Abbreviations: AM, Atractylodes macrocephala; IEC-6, intestinal epithelial cell 6; STIM1, stromal interaction molecule 1; STIM2, stromal interaction molecule 2; q-PCR, quantitative polymerase chain reaction; WB, whole blood.
Figure 6. The effect on Ca\textsuperscript{2+} pathways. IEC-6 cells were incubated with ingredients (total 20 μM per group) or not for 24 h. After incubation, IEC-6 cells were determined by flow cytometry and WB, n = 3. (A) The result of [Ca\textsuperscript{2+}]\textsubscript{cyt} concentration in cells. (B) The effects on TRPC1, PLC-γ1 and RhoA protein expressions in Ca\textsuperscript{2+} pathways. *, **, *** denote P < .05, P < .01, and P < .001 versus the control group. #, ##, ### denote P < .05, P < .01, and P < .001 versus combination group.

Abbreviation: IEC-6, intestinal epithelial cell 6; WB, whole blood; TRPC1, transient receptor potential 1; PLC-γ1, phospholipase C-γ1.
inflammatory cytokines, which play a vital role in the repair of intestinal epithelial cells. In this study, we found that atracylone, β-eudesmol, atracylenolide II, atracylenolide III, and the combination all had an anti-inflammatory effect for IEC-6 cells, but with different benefits in the anti-inflammatory cytokines IL-2, IL-10, and ODC. Only atracylenolide III and the combination of the 4 AM ingredients could significantly increase the expression of IL-2, IL-10, and ODC, while the combination of 4 AM ingredients was slightly better than all the individual AM ingredients. Therefore our result suggested that atracylenolide III had the best anti-inflammatory capacity in the 4 compositions, and the combination enhanced the anti-inflammatory activity.

Epithelial repair is the tight coordination of processes including migration, proliferation, and differentiation. 

So, proliferation and migration are key events during the epithelial repair. In the previous study, it has been shown that AM Koidz could promote IEC-6 cell migration, which was a dose-dependently process. Our study showed that atracylenolide III performed the best ability of IEC-6 cell migration in the 4 AM compositions and the combination of the 4 AM ingredients showed a better pro-cell motility and cell cycle for IEC-6 cells than any individual AM ingredients. In addition, cell proliferation depends on an orderly cell cycle process, our study pointed out that the 4 compositions and the combination not only increased the number of G1 phase cells, but also reduced the number of S phase cells. Thereinto, atracylon, and atracylenolide III had a stronger promotive effect on IEC-6 cells and the combination of the 4 AM ingredients intensified the change in the cell cycle process. Therefore, we believed that atracylenolide III played a major role in cell proliferation and migration, and the 4 ingredients had synergistic effects. Apart from that, polyamines play an essential role in the regulation of cell differentiation and proliferation and tissue repair, and the intracellular levels of polyamines are highly regulated by the activity of ODC. Song et al. found a significant increase of polyamines in IEC-6 cells with AM Koidz treatment, and thought the increase of polyamines was related to the promotional effect of AM Koidz on IEC-6 cell migration. In our study, we observed the level of ODC in IEC-6 cells was increased, so it suggested that the contribution of atracylenolide III and their combination to IEC-6 cells proliferation and migration was connected to polyamine.

It has been reported that AM and its some active ingredients can repair intestinal epithelium through the Ca\textsuperscript{2+} pathway. For example, the study of Song et al. revealed that the treatment with AM Koidz significantly stimulated the migration of intestinal epithelial cells through polyamine-Kv1.1 channel signaling pathway, when intestinal epithelium got an injury. Zeng et al. found that the polysaccharide of AM Koidz extracts stimulated the migration of intestinal epithelial cells via a polyamine-Kv1.1 channel activated signaling pathway, which facilitated intestinal injury healing. Further, another study provided additional evidence suggesting that atracylenolide I promoted cell migration and proliferation, increased polyamines content, raised cytosolic free Ca\textsuperscript{2+} concentration, and enhanced TRPC1 and PLC-γ1 expression in IEC-6 cells. However, there were few studies comparing the effects of the active ingredients of AM on the Ca\textsuperscript{2+} pathway. Therefore, we detected the effects of atracylon, β-eudesmol, atracylenolide II, atracylenolide III, and their combination to the Ca\textsuperscript{2+} pathway. As we expected, atracylenolide III significantly increased [Ca\textsuperscript{2+}]\textsubscript{cyt} cell concentration and STIM1 expression and the up-regulated TRPC1, PLC-γ1, and RhoA levels, and their combination strengthened the effect.

Our data suggested that the 4 compositions of AM promoted intestinal epithelial repair by promoting Ca\textsuperscript{2+} influx and up-regulating the cell [Ca\textsuperscript{2+}]\textsubscript{cyt} concentration, in particular, atracylenolide III exerted the most prominent effect. Moreover, our results highlighted a mutual facilitative interaction on the Ca\textsuperscript{2+} pathway among the 4 compositions and their combination was the most promising. However, the same type of research is still scarce, there is a lack of comparison of the research conclusions. In the future, we will take account of some aspects to research the effects of active components of AM and their combined use on intestinal epithelial repair.

In conclusion, our results demonstrated that atracylon, β-eudesmol, atracylenolide II, and atracylenolide III all promoted the IEC-6 cells’ abilities of proliferation, migration, and anti-inflammatoryatory functions, and increased the intracellular [Ca\textsuperscript{2+}]\textsubscript{cyt} concentration through the Ca\textsuperscript{2+} pathway, promoting intestinal epithelial recovery. Of these, atracylenolide III exerted the most prominent effect relatively and the improvement of the 4 ingredients combination use was better than using them alone. Therefore, we emphasized the contribution and treatment value of their combined use.

![Chemical structures of atracylon, β-eudesmol, atracylenolide II, and atracylenolide III.](image)

Figure 7. Chemical structures of atracylon, β-eudesmol, atracylenolide II, and atracylenolide III.
Methods

Reagents

The standards of the 4 active ingredients (atracylone, \(\beta\)-eudesmol, atractylenolide II, and atractylenolide III) were obtained from Chengdu Push Bio-Technology Co., Ltd. Atracylone standard, CAS No 6989 to 21 to 5, purity \(\geq 98\%\), white or light yellow crystal; \(\beta\)-eudesmol, CAS No 473 to 15 to 4, purity \(\geq 98\%\), white acicular crystal; atractylenolide II, CAS No 73069 to 14 to 4, purity \(\geq 98\%\), white crystal; atractylenolide III, CAS No 73030 to 71 to 4, purity \(\geq 98\%\), white acicular crystal. The chemical structures of those compounds are provided in Figure 7.

Preparation

The IEC-6 cell was purchased from the American Type Culture Collection (NO. 63139935). The cells were cultured with high-glucose Dulbecco’s modified Eagle’s medium (high-glucose DMEM) containing 100 units/mL penicillin, 100 \(\mu\)g/mL streptomycin, which facilitated intestinal injuries of 95% air-5% CO\(_2\) at 37°C. Cell subculturing was performed when the cells reached 80% to 90% coverage. The 16th to 19th passages of IEC-6 cells were used for this experiment.

The compound standards were weighed accurately and added to a volumetric flask (100 mL). Then the compound standards were dissolved in DMEM, sonicated, and the final volume was made up to 100 mL with DMEM. Finally, the solution was filtered through a 0.22 \(\mu\)m filter. The weight of the compound standards was determined by the required concentration of the test, and the formula is:

\[
g = C \times V \times M \times 1 \times 10^{-6}
\]

where \(C\): experimental concentration (\(\mu\)M); \(V\): the volume of a volumetric flask (0.1 L); \(M\): the molar mass of the compound (g/mol).

\(3-[4,5\text{-dimethylthiazol-2-yl}]-2,5\text{ diphenyl tetrazolium bromide (MTT)}\)

IEC-6 cells in the logarithmic phase were enzymatically digested to a single-cell suspension. The cells were plated at 2 \(\times\) 10\(^5\) cells per well to 6-well plates and allowed to attach. The complete DMEM separately containing various concentrations (0-160 \(\mu\)M) of \(\beta\)-eudesmol, atractylenolide II, atractylenolide III, and atracylone were added and incubated with IEC-6 cells for 24 h and 48 h. After culture, cell proliferation was assessed using an MTT assay, performed as previously described. Briefly, added 20 \(\mu\)L MTT (5 mg/mL) for 4 h. Thereafter, 150 \(\mu\)L dimethyl sulfoxide (DMSO) was used to solubilize the dark blue crystals for 10 min. The results were monitored at 490 nm in a microplate reader (Bio-Rad). Cell growth proliferation rate was calculated as \([A_{490\text{ nm}} \text{ (treated cells)} / A_{490\text{ nm}} \text{ (control cells)} - 1] \times 100\%\).

Orthogonal Experiment

According to the results of MTT, an L9 (3\(^4\)) orthogonal test was employed to choose the best combination of the 4 active ingredients. Each factor level is shown in Table 1 and the L9 (3\(^4\)) orthogonal table shown in Table 2. The control group was not given any intervention of the 4 active ingredients. Cell treatment was identical to the MTT assay described above and cultured for 24 h.

Following Experimental Groups

The following experiments were divided into 6 groups, containing the control group, atracylone group, \(\beta\)-eudesmol group, atractylenolide II group, atractylenolide III group, and the combination group (depended on the result of the orthogonal experiment) and the drug concentration was 20 \(\mu\)M.

Enzyme-Linked Immunosorbent

IEC-6 cells in the logarithmic phase were seeded in 6-well plates at a concentration of 2 \(\times\) 10\(^5\) cells per well and allowed to grow for 24 h. Then, 4 active ingredients and their combination were respectively added to the 6-well plate and cultured for 24 h. After this step, cell culture supernatants were collected for ELISA. ELISA assays were determined for IL-2, IL-10, and

| Table 1. Factors and Levels for the L9 (3\(^4\)) Orthogonal Table. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Levels | A Atractylon | B \(\beta\)-Eudesmol | C Atractylenolide II | D Atractylenolide III |
| 1 | 10 \(\mu\)M | 10 \(\mu\)M | 10 \(\mu\)M | 10 \(\mu\)M |
| 2 | 20 \(\mu\)M | 20 \(\mu\)M | 20 \(\mu\)M | 20 \(\mu\)M |
| 3 | 40 \(\mu\)M | 40 \(\mu\)M | 40 \(\mu\)M | 40 \(\mu\)M |

| Table 2. The L9 (3\(^4\)) Orthogonal Table. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Test number | A | B | C | D |
| 1 | 1 | 1 | 1 | 1 |
| 2 | 1 | 2 | 2 | 2 |
| 3 | 1 | 3 | 3 | 3 |
| 4 | 2 | 1 | 2 | 3 |
| 5 | 2 | 2 | 3 | 1 |
| 6 | 2 | 3 | 1 | 2 |
| 7 | 3 | 1 | 3 | 2 |
| 8 | 3 | 2 | 2 | 3 |
| 9 | 3 | 3 | 1 | 1 |
Table 3. Primer Sequences.

| Gene   | Primer sequences                        |
|--------|-----------------------------------------|
| STIM1  | F: GCACCTATTCGCTACTTC                    |
|        | R: GATTTGGTTCTCTCCCATGTC                |
| STIM2  | F: GAGAAACCAACAACCTCAAC                 |
|        | R: CTGACAGCACCACCTGATAA                 |
| β-actin| F: GGGAGATTACCTGCCC GGCTCCCTA          |
|        | R: GACICATCGTACCTCGCTGTCG              |

Abbreviations: STIM1, stromal interaction molecule 1; STIM2, stromal interaction molecule 2.

ODC by ELISA kits (Abcam) according to instructions. Briefly, samples were added to 96-well plates precoated with antibodies. After several incubation steps, washing procedures, and a final incubation with a chromogenic substrate, the result was measured with a microplate reader (Bio-Rad).

**Wound Healing Assay**

Wound healing assay migration was tested by a wound-healing assay. IEC-6 cells in the logarithmic phase were seeded in 6-well plates at a concentration of 2 × 10^5 cells per well and allowed to grow for 24 h, reaching a cover of 80%. The scratch was drawn perpendicularly with a 10 μL pipette tip. PBS was used to wash off damaged cells in plates. Subsequently, the complete medium (including 4 μg/mL mitomycin) with different active ingredients based on the above grouping information was treated for 24 h. Photomicrographs of the cell migration were taken with an Olympus inverted phase-contrast microscope (Japan). The relative wound recovery ratio of the cell was calculated as follows: relative wound recovery ratio (%) = [distance within scratch (0 h) − distance within scratch (24 h)] / distance within scratch (0 h).

**Cell Cycle Analysis**

Cell cycle analysis was detected by the BD Cyclestest Plus DNA kit. IEC-6 cells in the logarithmic phase were seeded in 6-well plates at a concentration of 2 × 10^5 cells per well and allowed to grow for 24 h. The respective medium was treated for 24 h with complete DMEM separately containing different active ingredients based on the above grouping information. After digestion with trypsin, 200 μL solution of ice-cold PI stain or PBS was added into 6-well plates for 10 min at the temperature 37°C. Finally, the cells were washed and the fluorescence was acquired with an Accuri C6 flow cytometer.

**Flow Cytometry for Ca^{2+}**

IEC-6 cells in the logarithmic phase were seeded in 6-well plates at a concentration of 2 × 10^5 cells per well and allowed to grow for 24 h. The respective medium was treated for 24 h with complete DMEM separately containing different active ingredients based on the above grouping information. Ca^{2+} concentration in cells was tested by flow cytometry. Briefly, the upper culture solution was discarded carefully and the remaining cells were washed twice with PBS. Then 5 μL fluo-3 AM (2 mmol/L) was added into 6-well plates for 45 min at the temperature 37°C. Finally, the cells were washed and the fluorescence was acquired with an Accuri C6 flow cytometer.

**Quantitative Polymerase Chain Reaction (q-PCR)**

The cell culture and the treatments were the same as that of flow cytometry. Total RNA was collected by TRIzol RNA extraction kit (Invitrogen) and RNA integrity and purity were determined by gel electrophoresis and NanoDrop 2000 spectrophotometry (Thermo). Reverse transcription was conducted using a reverse transcription kit (Invitrogen) as described by the manufacturer. Then the resulting cDNA was used for quantitative analysis by q-PCR, assessed by a Qubit spectrophotometer (Invitrogen). The primer sequences are shown in Table 3. Gene expression was normalized to β-actin.

**Whole Blood**

The cell culture and the treatments were the same as that of flow cytometry. The total protein was extracted from the cells. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrostatically to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk in TBS (50 mM Tris, pH 7.6, 0.9% NaCl, and 0.1% Tween-20) at room temperature, and then incubated overnight at 4°C with primary antibody against. The membranes were washed with TBS and then a secondary antibody with HRP labeled was combined. After washing again, the protein bands were visualized by a Western ECL Substrate (Bio-Rad) and the result was determined by Image Lab software (Bio-Rad).

**Immunofluorescence Staining**

The cell culture and the treatments were the same as that of flow cytometry. At the end of the culture time, cells were fixed using 3.7% formaldehyde. The slides then were blocked with 2% BSA for 30 min. A primary antibody against STIM1 (1:300) was added and incubated overnight. After 3 washes, a secondary antibody Alexa Fluor-594 (Invitrogen, USA) was added for 2 h. Finally, DAPI nuclei staining was performed. The slides were viewed and photographed under a DM2000 fluorescence microscope (Leica).

**Statistical Analysis**

All values were expressed as means ± SD, and SPSS 19.0 software was used for analysis. Differences in mean values were determined by one-way analysis of variance test. Then, multiple comparisons between the groups were determined using the
least significant difference’s multiple range test. P<.05 was accepted as statistical significance.

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Supplemental material for this article is available online.

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