Gut microbiota-derived 5-hydroxyindoleacetic acid from Pumpkin Polysaccharides supplementation alleviates colitis through Epac/Rap1 signaling activation

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Abstract

Aims

Polysaccharides from Pumpkin (*Cucurbita moschata* Duchesne) (PP) have many pharmacological activities, including anti-oxidant, immune, and intestinal microbiota regulation. These activities have provided some reminders of its potential therapeutic effect on ulcerative colitis (UC), but this has not yet been confirmed. This study explores the potential modulatory roles of gut microbial metabolites (5-hydroxyindole acetic acid, 5-HIAA) after PP treatment in protecting against UC and to elucidate the underlying molecular mechanisms.

Results

The average molecular weight of PP was $3.10 \times 10^5$ Da, and PP mainly comprised Mannose, Rhamnose, Galacturonic acid, Galactosamine, Glucose, and Xylose with molar ratios of 1.58:3.51:3.45:1.00:3.25:3.02. PPs (50, 100 mg/kg) could significantly resist dextran sodium sulfate (DSS) induced UC on C57BL/6 mice by improving gut microbiota dysbiosis and intestinal mucosal barrier function. Metabolomic profiling analysis showed that PP supplementation resulted in the enrichment of 5-HIAA, which exhibited individual and synergistic anti-UC activities. The results of the transcriptome and confirmatory experiments suggested that Rap1 pathway activation may participate in the anti-UC effect of PPs. 8-pCPT-2′-O-Me-cAMP (Epac/Rap1 signaling antagonist) showed similar anti-UC activity to PP and 5-HIAA, while the effect of ESI-05 (Epac/Rap1 signaling inhibitor) was opposite. Furthermore, exogenous treatment with 5-HIAA activated the Epac/Rap1 signaling pathway both *in vitro* and *in vivo*, which may be related to elevated cAMP levels in the colon.

Conclusions

This study revealed that PP could be developed to treat UC and other diseases associated with an imbalance in the intestinal microbiota. The gut microbiota-5-HIAA-Epac/Rap1 axis plays an important role in intestinal homeostasis and may provide a novel approach for the prevention of UC by manipulating gut microbial serotonin metabolites.

Introduction

Ulcerative colitis (UC) is an intestinal inflammatory disease with an incomplete etiology and pathogenesis that is long and prone to recurrent attacks. At present, a variety of drugs can be used to treat UC — including salicylates, hormones, and immunosuppressants. However, there are many problems, such as limited curative effects, long treatment cycles, high recurrence rates, and the frequent and serious accompanying complications [1]. The active components of traditional Chinese medicines (TCM) have many advantages — such as low toxicity and side effects, low cost, and large space for structural transformation. There is important research value and clinical significance in exploring natural products with low toxicity, high efficiency, and which are administered orally to treat UC. Plant polysaccharides have been proven to have various biological activities, including immune regulation; hypoglycemic, anti-tumor, hypolipidemic, anti-radiation, anti-bacterial, and antiviral activities; liver protection; other health functions; and a relatively low level of toxicity [2]. Many plant polysaccharides can regulate intestinal inflammation in UC by regulating inflammatory cytokines, intestinal microbiota, and the immune system and by protecting the intestinal mucosa [3,4]. These mechanisms are well-matched with the pathogenesis of UC — such as immune disorders, inflammation, oxidative stress, and gut dysbiosis.

The pumpkin (*Cucurbita moschata* Duchesne ex Poiret) (*C. moschata*) is a popular cultivated vegetable with a variety of promising health-promoting properties. Many beneficial nutritional and medicinal properties of various *C. moschata* extracts have been reported, including antioxidant, anti-diabetic, anti-obesity, anti-hypercholesterolemic, anti-bacterial [5], anti-prostate cancer, and intestinal microbiota regulatory activities [6]. Pumpkin polysaccharides (PP) are one of the primary functional and nutritional components derived from pumpkins, and exhibit anti-tumor, anti-oxidant, anti-bacterial, anti-diabetic, and anti-obesity properties [7]. Based on these pharmacological activities, we speculate that PP may have the potential to treat UC. Previously, we screened active ingredients from TCM to treat UC and recorded a number of outcomes [8–11]. As one of our achievements of screening active
natural anti-UC components, this study aims to explore the therapeutic effect of PP on dextran sodium sulfate (DSS) induced UC mice and to investigate the underlying mechanism of action.

In this study, we demonstrated that PP significantly mitigates disease severity and microbiota dysbiosis in UC mice. Gut microbiota is involved in PP-induced metabolic reprogramming of colonic tissues. Additionally, we identified a gut microbiota-derived 5-HT metabolite 5-hydroxindoleacetic acid (5-HIAA), which mediates the beneficial effects of PP on intestinal function via the activation of Epac/Rap1 signaling. Rap1 is a small GTPase that regulates integrin-mediated lymphocyte adhesion and migration, and is crucial for immune monitoring. Like Ras, Rap1 uses a small GTPase as a molecular switch that cycles between the activation of GTP binding and the deactivation of GDP binding [12]. This cycle is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Rap1 plays crucial roles in angiogenesis, vascular endothelial barrier stability, and vascular tension. After activating Rap1, it effectively protects the vascular endothelial barrier [13]. Previous in vitro studies have shown that cAMP analogs (8’-pcPT-2’-O-Me-cAMP) can specifically activate EPAC and then activate Rap1 [14]. Research on Rap1 in UC has rarely been reported, and research on its mechanism has been neither in-depth nor specific. Thus, our data provide new insights into the underlying mechanisms of PP in protecting against colitis, highlighting the potential application of PP and 5-HIAA in the prevention of UC by activating Rap1 and evaluating Rap1GTP levels.

Results

Chemical composition and anti-UC activity evaluation of PPs

The appearance and morphology of PPs were extracted and purified from pumpkins (Fig. 1A). As shown in Fig. 1B and Table S1, PPs exhibited the characteristic absorptions of polysaccharides at 3,401.82, 2,937.06, and 1,614.13 cm\(^{-1}\) for the O-H, C-H, and C-O stretching vibrations, respectively. The absorptions at 1,000–1,200 cm\(^{-1}\) were ascribed to C-O-C and C-O-H in a pyranose ring. The HPGPC spectrum showed that PP had a single symmetric peak, indicating that PPs are a homogenous polysaccharide (Fig. 1C) with a weight-average molecular weight (Mw) of 3.10 \(\times\) 10\(^5\) Da (Mw/Mn = 3.98). The monosaccharide composition of PPs mainly consisted of Mannose, Rhamnose, Galacturonic acid, Galactosamine, Glucose, and Xylose in molar ratios of 1.58:3.51:34.54:1.00:3.25:3.02 (Fig. 1D and Table S2), compared to the peaks of the monosaccharide standard (Fig. S1). Regarding the anti-UC effect, colon length in the model group was significantly shortened (Fig. 1E), whereas PPs and SASPs can significantly improve colon shortening (Fig. 1F). PPs (50 and 100 mg/kg) and SASPs (200 mg/kg) significantly reduced body weight loss (Fig. 1G) and DAI scores (Fig. 1H), particularly in the PP (100 mg/kg) group. The mucosal layer in the model group showed various pathological characteristics of UC, including irregular glands and numerous infiltrating inflammatory cells. PP (50 and 100 mg/kg) and SASP (200 mg/kg) treatment resulted in fewer infiltrated inflammatory cells, more complete tissue, and a relatively regular gland arrangement (Fig. 1I). SASPs and PPs significantly improved the histopathological scores for colonic inflammation (Fig. 1J). In addition, PPs and SASPs significantly inhibited the enlargement of the spleen as induced by DSS (Fig. S2A and B). However, PPs did not exhibit any effects on IL-1\(\beta\) and TNF\(\alpha\) levels in LPS and Nigericin-induced inflammatory model in vitro, suggesting that anti-UC effect of PPs may not be achieved through direct anti-inflammatory mechanisms (Fig. S2C and D).

The improvement of intestinal microbiota disorder of PPs

Analysis of the intestinal microflora of the colonic contents showed that 272 OTUs overlapped in all groups, whereas 297 OTUs appeared in the control and model groups. Meanwhile, 298 OTUs overlapped in the control and PP (100 mg/kg) groups, and 291 OTUs appeared in the control and SASP (200 mg/kg) groups (Fig. 2A). The exclusive OTUs for the control, model, PP, and SASP groups were 51, 3, 10, and 4, respectively. The total number of OTUs in the control, model, PP, and SASP groups was 365, 346, 354, and 344, respectively. In addition, PPs reversed the Feature, ACE, Chao1, Simpson, Shannon, and PD-whole_tree indices compared with the model group (Table S3). Principal component analysis (PCA) (Fig. 2B), principal coordinate analysis (PCoA) (Fig. 2C) and non-metric multidimensional scaling (NMDS) analysis (Fig. 2D) show that the control and model groups had completely isolated clusters. However, the PP group was more inclined towards the control group (Fig. S3C and D). Cluster analysis showed that three phyla (Bacteroidetes, Deferribacteres, and Proteobacteria) displayed a significant increase, and Campylobacterota decreased in relative abundance of the models at the phylum level (Fig. 2E). At the genus level, the relative abundances of the Bacteroides, Culuromicina, Mucispirillum, Escherichia-Shigella, and Alistipes increased in the model group. The abundance of Helicobacter decreased after DSS stimulation (Fig. 2F). However, PPs reversed these changes, indicating that PPs could improve and regulate...
the diversity of the intestinal microbiota towards a standard of normality. The result of heat map also show that the control and model groups had relatively far distance. However, the PPs was more inclined towards the control group (Fig. 2G).

The protective effect of PPs on the intestinal mucosal barrier

Consistent with the changes of the above bacteria, the cladogram, generated from the linear discriminant analysis effect size (LEfSe) analysis, showed distinct gut microbiota compositions among mice from all groups (Fig. S3A and B). The comparison of dominant bacterial taxa suggest that PPs significantly increased the relative abundance of k__Bacteria\_p__Firmicutes\_c__Clostridia\_o__Oscillospirales\_f__Oscillospiraceae\_g__Oscillibacter. Faecalibaculum_rodentium and Oscillospiraceae are two kinds of probiotic that may be closely related to maintaining intestinal epithelial homeostasis [15,16]. The result of GO analysis of intestinal microbiota show that the replication, recombination, repair and defense mechanisms may involved in the anti-UC effect of PP (Fig. 3A). In addition, the expression levels of the tight junction proteins (ZO-1, Occludin, and Claudin-1) were significantly reduced in the model group, indicating a disruption of intestinal barrier function (Fig. 3B). However, PPs (50 and 100 mg/kg) repaired the intestinal barrier by upregulating the expression of ZO-1 (Fig. 3C), Occludin (Fig. 3D) and Claudin-1 (Fig. 3E), indicating that PPs play a therapeutic role in repairing the intestinal barrier. To further determine whether there are potential associations between the alterations of gut microbiota and inflammatory cytokines, the correlation between the relative abundance of gut microbiota and the increased levels of ZO-1, Occludin and Claudin-1 were analyzed using correlation heatmap analysis (Fig. S3F). Mucispirillum was significantly positively correlated with ZO-1, Occludin and Claudin-1. Mucispirillum is a symbiotic bacterium in the intestine, which has been reported multiple times to have the effect of resisting colitis and protecting the mucosal barrier [17,18]. These results showed that PPs may improve intestinal mucosal injury by upregulating various bacterial strains, which always have protective effects on intestinal mucosa.

PP promotes the enrichment of gut microbiota-derived 5-HT metabolites

The volcano plot showed that PP feeding resulted in a dramatic alteration of metabolites, with a total of 14,006 metabolites having changed (8,388 up-regulated and 5,618 down-regulated) in the mouse colonic contents (Fig. 4A). PCA analysis exhibited a significant separation of clusters between the control and model groups. Meanwhile, the PP (100 mg/kg) group was inclined toward the control group, suggesting that PPs could improve the intestinal flora and tend to be normal (Fig. 4B). The KEGG analysis plot showed that serotonin related signaling pathway was the most significant metabolic pathway affected by PPs (Fig. 4C). We also found that amino acid metabolism plays an important role in predicting the metabolic pathways in mice with DSS-induced UC. Using LC-MS/MS, we also detected dozens of serotonin related metabolites and found that 5-HIAA was one of the most significantly increased metabolites in the colonic contents in response to PP feeding (Fig. 4D). In addition, 5-HIAA alone (10 and 20 mg/kg), or in conjunction with PPs, also had significant anti-UC effects, including improved colon length (Fig. 4E and F), body weight (Fig. 4G), and disease index (Fig. 4H), suggesting that PPs may exert a therapeutic effect by increasing 5-HIAA content after improving intestinal flora. Although there are many research results suggesting that increasing the concentration of 5-HIAA may have a positive correlation with the improvement of colitis [19–21], we provide direct evidence for the first time that 5-HIAA has the therapeutic effect on UC mice.

PP alters gut gene expression profile and activates the Epac/Rap1 signaling pathway

Genome-wide transcriptional profiling of colonic tissues was performed using RNA sequencing after PP treatment. PCA analysis indicates that PPs (100 mg/kg) were inclined toward the control group, suggesting that PPs could improve the gene expression profile, which tended to be normal (Fig. 5A). KEGG pathway analysis of the differentially-expressed genes showed that Ras and Rap1 were the most significantly enriched functional pathways in PP-treated mice (Fig. 5B). Meanwhile, the results of KEGG enrichment barplot showed that focal adhesion and the regulation of actin cytoskeleton were the most significantly enriched cellular processes (Fig. S4A). In terms of GO molecular functions, these differential genes are mainly involved in the regulation of protein binding, integral component of membrane, cell adhesion, inflammatory response and cell proliferation (Fig. 5C and Fig. S4B). These results also support the protecting role of PP on intestinal mucosal damage. The heat map shows PP could reverse the significantly high transcription of multiple genes associated with the Rap1 pathway inhibition [22], such as Rap1gapos, Rap1GAP, and Rap1gap2 (Fig. 5D). To confirm whether Epac/Rap1 signaling pathway was associated with an improved anti-UC effect, after multiple times of dosage explorations and optimizations, we found that Epac/Rap1 signaling antagonist (8'-pCPT-2'-O-
Me-cAMP) and inhibitor (ESI-05) exhibit a completely opposite effect. 8'-pCPT-2'-O-Me-cAMP feeding resulted in a more significant improvement of the anti-UC effect. However, when the mice were treated with ESI-05, various UC symptoms were further aggravated, including changes in colon morphology (Fig. 5E), length (Fig. 5F), body weight (Fig. 5G), and disease index (Fig. 5H).

5-HIAA protects against colitis via Epac/Rap1 activation

To determine whether Epac/Rap1 signaling activation is required for the protective effects of PPs and 5-HIAA on colitis, we examined the changes in the Epac/Rap1 signaling pathways after treatment with PPs and 5-HIAA in the DSS-induced colitis model introduced above. As expected, PP-fed UC mice exhibited Rap1 activation, which was manifested as an increase in Rap1GTP concentrations (Fig. 6A and B). Additionally, 5-HIAA increased Rap1GTP (Fig. 6C and D), followed by an increase in Raf-1 and β-catenin, as well as the activation of GSK3β and MEK phosphorylation, which may be related to the promotion of intestinal epithelial cell proliferation and mucosal repair activity (Fig. 6E-H). To further confirm the fact that 5-HIAA intervention exert their anti-UC effects by activating Epac/Rap1 pathway, we assessed the functional activity of 5-HIAA, 8'-pCPT-2'-O-Me-cAMP and tow Epac inhibitors (ESI-05 and ESI-09) on Epac/Rap1 signaling in human colon epithelial HT-29 cells. We found that 5-HIAA, 8'-pCPT-2'-O-Me-cAMP, ESI-05 and ESI-09 show no discernible toxicity on HT-29 cells under the concentration of 50 µM for 24 h incubation using CCK-8 detection. Interestingly, the levels of Rap1GTP show similar increases in levels of intensity after the treatment of 5-HIAA (20 µM) and 8'-pCPT-2'-O-Me-cAMP (2.0 µM), but not in ESI-05 (20 µM) and ESI-09 (20 µM) groups after treatment for 6 h (Fig. 6I and J), suggesting that 5-HIAA could activate the Epac/Rap1 signaling pathway. Considering the structural characteristics of Epac/Rap1 activators and the literature, we detected an increase in cAMP concentration after 5-HIAA treatment, which may be the mechanism by which 5-HIAA activates the Epac/Rap1 pathway (Fig. 6K). Collectively, these results suggest that 5-HIAA induces the expression of mucin proteins and exerts metabolites responsible for PP-induced anti-UC effects, at least in part, by elevating cAMP level and activating the Epac/Rap1 pathway (Fig. 6L).

Discussion

The pumpkin (C. moschata) is one of the most important vegetables in traditional agricultural systems worldwide [23] and has considerable nutritional and health-protective values. Some pumpkin polysaccharide components may play a role in the treatment of IBD [24]. Polysaccharides are the major chemical constituents of C. moschata and possess important pharmacological activities. However, currently, there are various extraction methods with no unified or standardized methodologies. The uncertainty and complexity of PP compositions limit their further development. Many extraction and purification methods have been developed to improve the total yield and purity of PPs. Several studies have isolated a series of polysaccharides from pumpkins and preliminarily determined their molecular weight and monosaccharide composition, including glucose, galactose, rhamnose, arabinose, galacturonic acid, glucuronic acid, mannose, xylose, and fucose, with Mw ranging from 10^3 to 10^6 Da [7]. Based on the methods of extraction, separation, and purification reported in other studies [25], we improved upon and designed a simple and efficient extraction method with high yield, high purity (> 97%), and relatively simple steps. We extracted high-purity PPs using an improved method and found that PPs are mainly composed of mannose, rhamnose, galacturonic acid, galactosamine, glucose, and xylose with Mw of 3.10 × 10^5 Da. These results are consistent with those obtained for polysaccharides isolated from C. moschata [26,27].

Natural plant polysaccharides always have low toxicity and possess immune and prebiotic activities, with positive effects in alleviating IBD by regulating intestinal flora imbalance, repairing intestinal barrier injury, and improving immunity [3,28]. Previous studies regarding polysaccharides from pumpkin fruit have mainly focused on their anti-oxidant, anti-diabetic, anti-tumor, immune regulatory, anti-coagulant, and other properties [7,26]. In addition to its repeatedly-proven antioxidant activity, PPs have also been shown to regulate intestinal microbiota disorders [27,29]. Dehydrodiconiferyl alcohol, a lignan compound isolated from C. moschata, display anti-UC effects on a DSS-induced colitis model by suppressing the production of TNF-α and IL-1β, as well as preventing weight loss and colon shrinkage [30]. These preliminary studies suggest that PPs exhibit anti-oxidant, anti-inflammatory, and intestinal microbiota effects, which may contribute to its therapeutic potential against UC. Although PP did not show significant anti-inflammatory activity in vitro, reasonable changes of many inflammatory factors (TNF-α, IFN-γ, IL-1β, IL-4, IL-6, IL-10, and IL-18) were still detected in the colon tissue of UC mice after PPs treatment. These results suggest that PPs might play an anti-inflammatory role through some mechanism after the metabolism of gut microbiota (Fig. S2E-K). The correlation between the relative abundance of gut microbiota and these inflammatory levels were analyzed using correlation heatmap analysis (Fig. S3E).
IL-4 was significantly positively correlated with the increased abundance of Mailhella and Lachnospiraceae_NK4A136_group after PPs treatment. These results expand the application range of PPs’ anti-inflammatory activity. The repair of the intestinal mucosa and the regulation of intestinal microbiota are at least the two anti-UC mechanisms of PPs.

The diversity and abundance of intestinal microbiota are important indicators that reflect the composition of the intestinal microbiota and intestinal health status [31]. At the phylum level, Firmicutes and Bacteroides were the dominant phyla in the intestinal microbiota of the control mice, and Firmicutes/Bacteroidetes ratio is always regarded as a hallmark of UC. Our results are consistent with those of other studies [32,33]; the abundance of Firmicutes/Bacteroidetes ratio in mice was always reduced after DSS administration, whereas PPs could reverse this ratio and make it closer to the control group. In addition, the enrichment of Deferribacteres [34], and Proteobacteria [35,36] have been reported to associate with colitis and intestinal barrier injury. The relative abundance of Campylobacterota often shows a decreasing trend after DSS intervention [37]. These results were consistent with the increase of their relative abundance after DSS administration in our results and PPs could reverse these changes. As for the main genus-level changes, Bacteroides and Escherichia-Shigella commonly exhibit a higher relative abundance in IBD individuals and further led to severe colitis [38]. We also detected an increase in the relative abundance of them in the model group. In addition, two probiotics, the Faecalibaculum_rodentium and Oscillospiraceae play important roles in the regulation and stabilization of the intestinal microbiota, and the abundance of these two bacteria always decrease in UC mice after DSS stimulation [15,16]. Our results showed that PPs and SASPs could significantly reverse the changes in these bacteria after DSS treatment, suggesting that variations in the abundance of these bacteria may have mainly contributed to the anti-UC effects of PPs.

Tryptophan metabolism is dysregulated in IBD and can be metabolized by commensal microbiota to produce multiple indole metabolites, including 5-HIAA, with diverse effects on mucosal immunity and homeostasis [39]. Serotonin signaling, as one of the metabolic pathways of tryptophan, may influence the intestinal immune response by modulating the gut microbiota composition, resulting in increased susceptibility to colitis [40]. Serotonin (5-HT) is produced by the EC cells and by the serotonergic neurons of the myenteric plexus within the bowel. Upon release, 5-HT is inactivated by the serotonin reuptake transporter and is broken down into 5-HIAA. 5-HT influences the immune response and intestinal inflammation [41]. Although enhanced levels of 5-HIAA have been reported in several studies of various colon inflammatory conditions [40,42], research on the effect of 5-HIAA in UC mice is scarce. In this study, we found for the first time that 5-HIAA alone had a significant anti-UC effect during the validation of the activity of a large number of metabolites obtained from the metabolomics results. In addition, we also discovered and validated multiple metabolites with similar anti-UC activities, such as propionic acid, 2-hydroxyacetic acid, indole-2-carboxylic acid, 5-hydroxyindole-3-acetic acid, L-alanyl-L-proline, cholic acid, and phenylbutyric acid — among others.

With the exception of the ability to adjust the abundance and diversity of intestinal flora, natural plant polysaccharides usually have the ability to regulate the abnormal expression of tight junction proteins so as to repair intestinal barrier damage [3]. After careful analysis of the colon tissue transcriptome, we found that, with the exception of some common classical signaling pathways, Rap1 related pathways may be most closely related to the anti-UC effect of PPs [43]. Rap1 plays a role in diverse processes such as cell proliferation, adhesion, differentiation, and embryogenesis. After the activation of Rap1, Rap1GTP is transferred to the cell membrane and exerts various pharmacological effects by affecting multiple downstream signaling pathways, such as activating the common Raf-1/B-Raf-MEK pathway to promote intestinal epithelial cell proliferation and that activating the GSK3β/β-catenin pathway plays a role in repairing the mucosal barrier [44–46]. Therefore, the upregulation of Rap1GTP is considered a key factor in achieving various therapeutic effects, including the treatment of UC. Some reports have suggested that serotonin activates Rap1 related pathways to promote cell adhesion and other cellular activities [47]. As a metabolite and structural analog of serotonin, we speculated and preliminary confirmed that 5-HIAA may also have similar activities. cAMP is an endogenous mechanism that downregulates the inflammatory response and prevents the progression of the acute inflammatory response to chronic inflammation and associated tissue destruction [48]. We preliminary confirmed, for the first time, that 5-HIAA activates the Rap1 pathway, which may be related to an increase in cAMP concentration.

We found and preliminarily confirmed the therapeutic potential of PP in UC and other disorders related to intestinal microbiota disturbances, which has great originality and expands the scope of C. moschata for the treatment of inflammatory diseases. Plant polysaccharides usually have low toxicity and C. moschata is a common food widely consumed worldwide, suggesting the potential value of PPs for further development [7]. In addition, we identified a new active metabolite (5-HIAA) and a new therapeutic modality (Rap1 activation) for UC after improving gut microbiota disorders. This study provides a new explanation for the
mechanism of action of gut microbiota regulation in the treatment of UC. However, there are still many unsolved problems and shortcomings present in this study. For example, no systematic toxicological studies have evaluated the toxicity of PPs. Moreover, there are various extraction methods for PPs, and there remains a lack of unified and fixed extraction processes. PPs obtained by our extraction method showed the advantages of high yield, high purity, and process stability. PPs showed significant anti-UC activity, which warrants further study of its active monomer components and pharmacological mechanisms. Finally, the mechanism of action has not yet been fully elucidated upon and more data from transgenic animal or human studies are needed to fully confirm these findings.

In conclusion, we extracted, isolated, and identified the main polysaccharide components of Pumpkin (PPs). PPs protect against DSS-induced colitis by improving intestinal barrier function and microbial dysbiosis. Meanwhile, PPs promotes the enrichment of the microbiota-derived 5-HT metabolite 5-HIAA, which induces the elevation of cAMP and activation of the Epac/Rap1 signaling pathway, contributing to the effects of PPs on the improvements of intestinal functions. These findings suggest that PPs, 5-HIAA, and Epac/Rap1 activators might be used to treat UC and other intestinal dysfunctions.

**Materials and methods**

**Reagents**

DSS was purchased from MP Biomedicals (USA), as previously reported [10]. The antibody against Rap1GTP was purchased from New East Biosciences (Wuhan, China). ESI-05 was purchased from GlpBio (USA). Antibodies of Raf-1, Phospho-GSK3β (p-GSK3β), β-catenin and Phospho-MEK (p-MEK) were bought from Abcam (China). The antibodies against ZO-1 (AF8394), occludin (AF7644), and claudin-1 (AF6504) were purchased from Beyotime Biotechnology (Shanghai, China). 8'-pCPT-2'-O-Me-cAMP was purchased from Santa Cruz Biotechnology, Inc. 5-HIAA was sourced from Shanghai Macklin Biochemical Co., Ltd. cAMP was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

**Extraction, purification and identification of PP**

High-purity PPs were isolated and purified from pumpkins in our laboratory according to a predesigned flow chart (Fig. S5). Briefly, 10 kg worth of pumpkins was processed by water extraction, concentration, ethanol precipitation, and degreasing. Finally, PPs were obtained by freeze-drying an aqueous solution. Fourier-transform infrared (FT-IR) spectroscopy was used to identify characteristic functional groups of the PPs. The PP powder was dried prior to tabletting with KBr powder and analyzed using FT-IR spectroscopy (FTIR-650, GangDong SCI. & TECH. CO., LTD, Tianjin, China). For the component analysis, PPs and standard samples were derived from 1-phenyl-3-methyl-5-pyrazolone before detection. High-performance liquid chromatography HPLC (Agilent 1100, G1315B DAD) was performed using a ZORBAX Eclipse XDB-C18 (4.6 mm × 250 mm, 5 µm) under the best liquid-phase conditions. At last, high performance gel permeation chromatography (HPGPC) was carried out using an Ultrahydrogel™ Linear column (300 mm × 7.8 mmid × 2; Waters Corporation, USA) so as to evaluate the molecular weight distribution of PPs. The mobile phase was 0.1 N NaNO₃, with a flow rate of 0.8 mL/min.

**Animal experiments**

All animals received humane care following the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of First Affiliated Hospital of Zhejiang University (approval No. 2018-023). Following the commonly used methodology, all C57BL/6 SPF mice (20 ± 2 g) were free to drink 3% DSS solution for seven days, except for the control group. Body weight loss and disease activity index (DAI) were evaluated daily in a blinded manner (Table S4). Pathological changes were observed and the histological score was evaluated according to the pathological evaluation criteria (Table S5). Based on a large number of previous studies, as well as our own pre-experiment, the optimal dosages of PPs were chosen as 50 and 100 mg/kg [49,50]. All surgical processes were similar to those performed with the same ethical approval number:2018-023. For the treatment with 5-HIAA, the mice were randomly divided into six groups of six mice each: control, model, salicylazosulfapyridine (SASP, 200 mg/kg), 5-HIAA (10 mg/kg), 5-HIAA (20 mg/kg), and 5-HIAA (10 mg/kg) + PP (50 mg/kg). For the treatment with Epac/Rap1 signaling antagonist (8'-pCPT-2'-O-Me-cAMP) and inhibitor (ESI-05), mice were treated with 0.3, 0.6 mg/kg/day and 2.0, 4.0 mg/kg/day intragastrically [49].

**The evaluation of anti-inflammatory activity in THP-1 cells**
As we previously reported [11], THP-1 cells were inoculated into 24 well plates (5 × 10^5/well) and 0.5 µg/mL LPS (Sigma, USA) was added to stimulate cells for 4 h. Different non-toxic concentrations of PP (2.5, 5.0, 10 µg/mL) and MCC950 (5 µM, NLRP3 inhibitor) were then added for 1 h. Subsequently, 10 µM Nigericin (NLRP3 activator) were added and treated for 1 h. The supernatant was collected for ELISA.

**Gut microbiota detection and analysis**

This experiment was commissioned out to Biomarker Technologies Co., Ltd. (Beijing, China) for the conduction and analysis. DNA was extracted using the TGuide S96 Magnetic Soil/Stool DNA Kit (Tiangen Biotech (Beijing) Co., Ltd.) according to the manufacturer's instructions. Universal primer sets 27F: AGRGTTTGATYNTGGCTCAG and 1492R: TASGGHTACCTTGTTASGACTT were used to amplify the full-length 16S rRNA gene from the genomic DNA extracted from each sample. After a series of standard operating procedures for amplification, quantification, purification, and sequencing, the raw reads generated from the sequencing were filtered, demultiplexed, and analyzed.

**Non-targeted metabolomics**

The detection and analysis of non-targeted metabolomics were performed by LC-Bio Technology Co., Ltd. (Hang Zhou, China). The collected colon samples were thawed on ice, and metabolites were extracted following the standard extraction procedure. All samples were analyzed using an LC-MS system under fixed and validated chromatographic conditions. A high-resolution tandem mass spectrometer — Triple-TOF-5600 plus (SCIEX, UK) — was used to detect metabolites in both positive and negative ion modes. The online KEGG, HMDB database was used to annotate the metabolites by matching the exact molecular mass data (m/z) of samples with those from the database. Student’s t-tests were conducted to detect differences in metabolite concentrations between the two phenotypes. Supervised PLS-DA was conducted using MetaX so as to discriminate different variables between groups.

**RNA extraction, library construction and bioinformatics analysis of RNA-seq**

Colon tissues were collected and the total RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After a series of quantitation, integrity evaluation, purification, fragmentation, reverse transcription, and amplification steps, 2×150bp paired-end sequencing (PE150) was performed using an Illumina Novaseq 6000 (LC-Bio Technology Co., Ltd., Hangzhou, China). The differentially expressed mRNAs were selected with a fold change >2 or <0.5 and with a parametric F-test to compare nested linear models (p < 0.05) using the R package edgeR.

**LC-MS/MS detection of cAMP and 5-HIAA**

LC-MS/MS was used to detect cAMP and 5-HIAA concentrations, as previously described [51,52]. Liquid chromatography was performed using a Prominence® HPLC system (Shimadzu, Tokyo, Japan), comprising a solvent delivery unit LC-20AD, an autosampler SIL-20AC, and a column oven CTO-20AC. Mass spectrometry was performed using an API 4500 QTRAP Tandem Mass Spectrometer (AB Sciex, Toronto, Canada) with a Turbo V® ion source. Separation was conducted on a SunFire™ C18 column (3.0 mm × 100 mm, 2.5 µm) with the column temperature set to 30°C. An isocratic mobile phase consisting of solvent A (0.1% formic acid in water) and solvent B (acetonitrile) at a volume ratio of 80:20 was delivered at a flow rate of 0.4 mL/min. Measurements were conducted on a mass spectrometer coupled with an ESI interface in negative mode. For cAMP, 5-HIAA, and 5-HICA (internal standards, IS), the various MRM transitions were m/z 328.1→134.3, m/z 191.9→146.1, and m/z 178.1→104.2. Both Q1 and Q3 mass detection was operated in unit resolution, and the dwell time was set at 200 ms. Other optimized parameters of ion source were as follows: collision gas at 6 psi, curtain gas at 20 psi, gas 1 at 55 psi, gas 2 at 60 psi, ion spray voltage at -4,500 V, and temperature at 550°C.

**Western blot analysis and immunohistochemistry (IHC) detection**

Following previously described methods [8], proteins in colon tissues or HT-29 cells were quantified and normalized to equal levels. All protein samples were separated and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with the corresponding primary and secondary antibodies. An electrochemiluminescence instrument was used to detect the grey values of the protein bands. For IHC detection, after a series of deparaffinization and rehydration processes, the colonic sections were blocked with 5% bovine serum albumin for 30 min at room temperature and then washed with PBS. All tissue
sections were incubated with the primary antibody ZO-1, occluding, and claudin-1 overnight at 4°C. The slides were washed three times with PBS and Alexa Fluor488 secondary antibody (Invitrogen) for 2 h.

**Epac/Rap1 activation in HT-29 cells**

HT-29 cells were purchased from the National Collection of Authenticated Cell Cultures of China and inoculated into 24 well plates (5 × 10^5/well). In order to verify the effect of different concentrations of 5-HIAA on Rap1 signaling pathway in HT-29 cells, different concentrations of 5-HIAA (0.25, 0.5, 1.0 µM), 8-pCPT-2’-O-Me-cAMP (0.25, 0.5, 1.0 µM) and ESI-05 (0.25, 0.5, 1.0 µM) were added for 24 h. Rap1 pull-down experiments were performed according to the reported method and the manual of Rap1GTP antibody [47]. Briefly, HT-29 cells were lysed in ice-cold lysis buffer containing a protease inhibitor cocktail for 10 min. After centrifugation at 10,000 g for 10 min at 4°C, GST-Ral guanine nucleotide dissociation stimulator (RalGDS)-raf-1 ras-binding domain coupled to glutathione-Sepharose beads was added to the supernatants and incubated for 1 h at 4°C. The beads were then washed thrice in lysis buffer, and the bound proteins were eluted with Laemmli sample buffer. Finally, Rap1GTP in the total cell lysates and precipitates was detected by western blot analysis using a mouse anti-Rap1GTP mAb.

**Statistical analysis**

All data except, intestinal microbiota determination, were presented as Mean ± SD of the three experiments; the statistical significance was calculated via one-way ANOVA following post hoc Tukey’s test using GraphPad 7.0 software. At p < 0.05, the results are considered statistically significant.

**Declarations**

**Author's contributions**

Qiao Zhang, Qi Wang, and Xiaodong Li are responsible for extracting the contents of separation and partial structural identification; Minglan Wu and Xia Wu are responsible for the development of animal experiments and the revision of manuscripts; Qingwei Zhao are responsible for revising manuscripts and providing experimental materials; Weifen Li, and Xingjiang Hu, as corresponding authors, provided financial support, organized all research, and drafted and revised the manuscript.

**Ethics approval and consent to participate**

All animals received humane care following the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of First Affiliated Hospital of Zhejiang University (approval No. 2018-023)

**Consent for publication**

All the listed authors have agreed to all the contents in the manuscript and the submission.

**Availability of data and materials**

Because of the sensitive nature of the data collected for this study, all the metadata are available through a request for external collaboration and upon approvals of a letter of intent.

**Competing Interests**

All authors reported that there's no declarations of interest.

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References


Figures

Figure 1

Chemical composition analysis, activity evaluation and diversity analysis of differential microbial communities in DSS-induced UC mice. (A) The appearance and morphology of PPs; (B) FT-IR spectrum of PPs; (C) HPGPC spectrogram of PPs; (D) Monosaccharide composition spectrum of PPs; (E) Colon length after PP treatment; (F) Statistical results of colon length in each group; (G) The curves of body weight changes; (H) The curves of DAI changes; (I) Representative pictures of HE staining in DSS-induced mice; (J)
Morphology score of colon sections. Each value was expressed as mean ± SD (n = 6). *p < 0.05, **p < 0.01 and ***p < 0.001 vs control; *p < 0.05, **p < 0.01 and ***p < 0.001 vs model.

Figure 2

The diversity analysis of differential microbial communities in DSS-induced UC mice. (A) A Venn diagram of the intestinal microflora; (B) Principal component analysis; (C) Principal coordinate analysis; (D) Non-metric multidimensional scaling; Clustering analysis of fecal microbiota among groups at the phylum (E) and genus (F) levels; (G) The result of heat map analysis. Each value was expressed as mean ± SD (n = 6).
Figure 3

PPs repaired the intestinal barrier of DSS-induced colon injury. (A) The result of GO analysis of intestinal microbiota. (B) Representative pictures of ZO-1, Occludin and Claudin-1 staining in DSS-induced mice; Quantitative results of relative protein expression ratios of ZO-1 (C), Occludin (D) and Claudin-1 (E). Each value was expressed as mean ± SD (n = 6). ###p < 0.001 vs control; *p < 0.05, **p < 0.01 and ***p < 0.001 vs model.
Figure 4

The results of metabolomic analysis in different groups. (A) Volcanic plot of metabolite changes; (B) Principal component analysis; (C) KEGG enrichment analysis; (D) Quantitative results of 5-HIAA; (E) Colon morphology and length of 5-HIAA treated mice; (F) Quantitative results of colon length; (G) Body weight changes and (H) disease active index after 5-HIAA treatment. Each value was expressed as mean ± SD (n = 6). **p < 0.01, ***p < 0.001 vs control; *p < 0.05, **p < 0.01 and ***p < 0.001 vs model.
Figure 5

The results of transcriptional analysis in different groups. (A) PCA analysis; (B) KEGG enrichment analysis; (C) Scatter plot of GO enrichment; (D) Heat map of gene distribution related to Rap1 pathway; The changes of colon morphology (E), colon length (F), body weight (G), and disease active index (H) after the treatment of 8’-pCPT-2’-O-Me-cAMP and inhibitor ESI-05. Each value is expressed as mean ± SD (n = 6). ### p < 0.001 vs control; * p < 0.05 and ** p < 0.01 vs model.
Figure 6

Epac/Rap1 signaling activation was required for the intestinal mucosal protection of PP and 5-HIAA. (A) Effect of PPs on Rap1GTP content and (B) quantitative results; (C) The effect of 5-HIAA on Rap1GTP and downstream proteins of Raf-1, p-MEK, p-GSK3β, and β-catenin; The protein quantification result of Rap1GTP (D), Raf-1 (E), p-MEK (F), p-GSK3β (G), and β-catenin (H); (I) The changes of Rap1GAP levels in HT-29 cells after the treatment of 5-HIAA, 8’-pCPT-2’-O-Me-cAMP, ESI-05 and ESI-09. (J) The protein quantification result of Rap1GTP on HT-29 cells; (K) The increase of cAMP concentration after 5-HIAA and PP treatment on the tissues of UC mice; (L) Mechanism of PPs and 5-HIAA action on UC mice. Each value was expressed as mean ± SD (n = 6). #p < 0.05, ###p < 0.001 vs control; *p < 0.05 and ###p < 0.001 vs model.

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