

Effects of *Potentilla Fulgens* as Prophylactic Agent in Intestinal Ischemia Reperfusion Injury

Efectos de *Potentilla fulgens* como Agente Profiláctico
en la Lesión por Isquemia-Reperusión Intestinal

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SUMMARY: The purpose of this study, ischemia reperfusion injury in rats, *Potentilla fulgens* is to investigate the protective effects. Wistar albino rats (n= 30) weighing 180-220 g were used in the experiment. Group 1 animals underwent sham laparotomy without ischemia-reperfusion injury. Group 2 animals underwent laparotomy and occlusion of superior mesenteric arteries for 30 min followed by 20 min of reperfusion without pretreatment. The *Potentilla fulgens* group received 400 mg/kg/day *Potentilla fulgens* intraperitoneally 5 days before Ischemia-reperfusion injury. There was a significant difference between the group with ischemia-reperfusion group *Potentilla fulgens* (p<0.0001). In statistical analysis of the MDA level, data were obtained after a respective measurement in all groups. *Potentilla fulgens* group with ischemia-reperfusion group was a significant decrease in MDA (p<0.001). In the period after ischemia-reperfusion, marked PCNA immunoreactivities were observed in the nuclei of crypt and villus cell. In ischemia reperfusion group, the number of PCNA immunoreactivity is quite advanced and they extended throughout the middle part of the intestine folds. The number of TUNEL-positive nuclei were also developed. In ischemia-reperfusion plus *P. fulgens* group, the intestinal epithelium with only a few PCNA immunoreactive nuclei. TUNEL positive nuclei were noted in the gut lumen and mucosal close differentiated goblet cells. We showed that *Potentilla fulgens* extract significantly prevented mucosal lesions caused by intestinal ischemia-reperfusion.

KEY WORDS: Intestinal ischemia; Proliferating Cell Nuclear Antigen (PCNA); Rat; Tunnel method.

INTRODUCTION

Intestinal ischemia-reperfusion (I/R) injury is essentially an inflammatory response process and is a significant problem in abdominal aortic aneurysm surgery, small bowel transplantation, cardiopulmonary bypass, strangulated hernias and neonatal necrotizing enterocolitis (Collard & Gelman, 2001). Ischemia-reperfusion injury (IRI) can be attributed to many factors such as the release of free oxygen radicals and consecutive lipid peroxidation, cell death by apoptosis or necrosis, inflammatory cytokines, and damage to the microvasculature (Granger *et al.*, 1981; Bodwell, 1989; Carden & Granger, 2000; Mallick *et al.*, 2004). Although the definitive pathophysiology regarding intestinal I/R injury still remains obscure, it is generally believed that oxidative stress mediators such as Reactive Oxygen Species (ROS), polymorphonuclear neutrophils and Nitric Oxide (NO) are suggested to play an important role (Murry *et al.*, 1986). *Potentilla fulgens* Lodd. is an alpine plant of Western Himalayas that is consumed in all parts of

the world for its promising medicinal properties. Pharmacologically, the aerial and root portions of the plant are reported to have antioxidant (in in vitro models) (Kaul *et al.*, 2010), antitumor (Syiem *et al.*, 2003), hypoglycemic and antihyperglycemic activities (Syiem *et al.*, 2009). Recently gastroprotective activity of ethanolic root extract of *P. fulgens* has also been reported (Laloo *et al.*, 2013). The general signs and symptoms of toxicity, food water intake and mortality rates of animals were observed for 72 h post-treatment. From these observations, LD50 was calculated using SPSS software (Tangpu *et al.*, 2014). Proliferating Cell Nuclear Antigen (PCNA) is a significant cell-cycle regulated nuclear protein for DNA-polymerase, the PCNA-labeled nuclei had been shown to identify cells in the late G1 and early S phases of the cell cycle, as well as cells undergoing DNA repair (Chen *et al.*, 2005). The purpose of this study, ischemia reperfusion injury in rats, *Potentilla fulgens* is to investigate the protective effect.

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MATERIAL AND METHOD

Wistar albino rats (n= 30) weighing 180–220 g were used in the experiment. The under rats were kept under specific pathogen-free conditions with 12-hour light/dark cycles and were given food and water ad libitum. All animal experiments were performed in accordance with our institutional guidelines after obtaining the permission from the laboratory animal committee. Anesthesia was induced by intramuscular injection of 25 mg/kg Ketamine hydrochloride +Xylazine.

Animals were randomly divided into 3 groups of 10 rats each. The sham group underwent sham laparotomy without Ischemia-Reperfusion injury. The Ischemia-Reperfusion injury group served as the control group. These animals laparotomy and occlusion of superior mesenteric arteries for 30 min followed by 20 min of reperfusion without pretreatment. The *Potentilla fulgens* group received 400 mg/kg/day *Potentilla fulgens* intraperitoneally 5 day before ischemia-reperfusion injury. Ten to fifteen minutes after general anesthesia, the peritoneum of each rat was opened for 30 min. The arteria mesenterica superior was clamped to create an ischemia, followed by 20 were obtained for MDA and histopathological examination. The samples of intestine tissue were fixed immediately formaldehyde 10% embedded in paraffine wax, sectioned serially at 4–6 µm and stained with hematoxyline-Eosin (H-E). One section from each rat was graded blindly and semi-quantitative, histological evaluations were graded from 0 to 5 by a single observer, according to the index of Park *et al.* (1990) (Table I).

At reperfusion destruction, an MDA level that indicated the lipid peroxidation was calculated as nanomole per tester's 1 g of duodenum tissue (Tangpu *et al.*). These tissue samples were homogenized after 0.5 ml, of 10% and 4 ml of 5% trichloroacetic acid were applied to them (all reagents and compounds were obtained from Sigma Chemical Co, St. Louis, Missouri, USA). They were centrifuged at 4000 rpm. A 1ml sample is taken and mixed with 1 ml of 67% thiobarbituric acid and heated for 10 min. The absorbance value was read with 532-nm spectrometry, and its nanomole value for 1 g tissue was found.

Immunohistochemical staining: The tissues were put into a formalin solution for fixation and then embedded in paraffin wax. Then they were cut into 4–6 mm sections on positively charged glass slides. Sections were deparaffinized with xylene, followed by immersion in graded alcohol for dehydration and incubation with EDTA (pH= 8.0, Merck, Germany) for 5+4+3 minutes in a microwave oven (750 Watt) for antigen retrieval. Next, sections were incubated for 20 min in 3% H₂O₂/Methanol to block endogenous peroxidase activity, then rinsed in phosphate-buffered saline (PBS) for 5 min three times. The sections were later incubated with a blocking solution (normal goat serum, Invitrogen, Carlsbad, CA). Slides were then incubated overnight with primary antibodies, Proliferating Cell Nuclear Antigen (PCNA) monoclonal antibody from Labvision Inc., Fremont, CA, USA. After washing in PBS, the sections were treated with labeled-streptavidin kits (Invitrogen, Carlsbad, CA). The reaction was visualized by incubating the sections for 7 min in a 0.1% 3,3 diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate kit, Invitrogen, Carlsbad CA). Finally, the sections were counterstained with Hematoxylin (Sigma) and covered. Immunohistochemistry positive staining was defined as the presence of a brown color detection chromogen (DAB) on the edge of the hematoxylin-stained cell nucleus distributed within the cytoplasm or plasma membrane of the cells and assessed by light microscope.

Tunnel technique: Anti-proliferating cell nuclear antigen (PCNA) antibody was employed for detection of the proliferation rate of enterocytes. Sections taken to distilled water were then washed in PBS 2x5 min sections was kept in freshly prepared permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) 8 min on ice. then washed in PBS 2x5 min and Added TUNEL Mixture (diluted 1:1 with TUNEL Dilution Buffer) on sections in the dark in humidified chamber for 1 at 37 °C. washed in PBS 2x5 min, As a substrate solution, Fast Red (Roche) is applied on sections. Sections were counterstained with Hematoxyline, Mounted with Kaiser's Glycerol Gelatine (Roche).

Table I. Scala for ischemic lesions (Histologic grading).

Grade 0	Normal morphology
Grade 1	Sub-epithelial edema and partial separation of apical cells
Grade 2	Moderate lifting of enterocytes from the tips of the villi
Grade 3	Lifting of enterocytes from both the tips and the sides of the villi (including superficial crypts)
Grade 4	Partial mucosal necrosis of the lamina propria
Grade 5	Total mucosal necrosis

RESULTS

In statistical analysis of histopathological findings, the data was obtained after grading assesment of the mucosal damage in all groups. The nonparametric Kruskal Wallis ANOVA procedure and a multiple comparison procedure based on the Tukey test has been used to analyse the histopathological scores ($p < 0.05$) (Table II). In statistical analysis of MDA level, data was obtained after a respective measurement in all groups.

There was a significant difference between the group with ischemia-reperfusion group *Potentilla fulgens* ($p < 0.0001$).

Potentilla fulgens group with ischemia-reperfusion group was a significant decrease in MDA ($p < 0.001$). Intestinal segments from the rats in the sham group displayed a normal microscopic appearance. The mucosa contained intact villi, crypts and submucosa; muscular layers were unaffected (Fig. 1A). Histopathological examination of duodenal and jejunum border obtained after I/R, revealed histopathological changes. These changes were represented desquamation and distortion of some villi epithelial sloughing and inflammatory cellular infiltration mainly polymorphonuclear cells, the tips of the villi and irregular loss of brush border in some areas and edema space in lamina propria region were observed. Dilatation and hemorrhage of blood capillaries, in connective tissue (Fig. 1B). Ischemia-reperfusion + fulgens group showed that the villi and the crypts were almost similar to those of the control group. Peeling at the villi apex was observed. Overall, the villar structure was well preserved. Decreased capillary congestion and sparse inflammatory cell infiltration was noted. The crypts of Lieberkuhn appeared as invaginations of the mucosa between the bases of the villi. Proliferating cell nuclear antigen staining showed PCNA positive nuclei mostly distributing in the nuclei of crypt cells, In ischemia reperfusion group, the number of PCNA immunoreactivity is quite advanced and they extended throughout the middle part of the intestine folds; The number of TUNEL-positive nuclei were also developed.

In the period after ischemia-reperfusion, marked PCNA immunoreactivities were observed in the nuclei of crypt and villus cell. In ischemia reperfusion group, the number of PCNA immunoreactivity is quite advanced and they extended throughout the middle part of the intestine folds; In ischemia-reperfusion + fulgens group, the intestinal epithelium with only a few PCNA immunoreactive nuclei by TUNEL positive nuclei were noted in the gut lumen and mucosal close differentiated goblet cells.

Table II. The data of histopathological assessments according to Park *et al.* (1990)'s grading of the mucosal damage.

Indices (Rat) n= 10	Sham group	Ischemi-reperfusion group	Ischemi-reperfusion + <i>PotentillaFulgens</i> group
1	0	5	1
2	0	4	1
3	0	4	2
4	0	4	1
5	0	5	2
6	0	3	1
7	0	5	2
8	0	4	2
9	0	5	2
10	0	5	2
Mean	0	4.3	1.6

DISCUSSION

Intestinal ischemia-reperfusion can have significant effects in many clinical situations. Parks & Granger (1986) reported that tissue lesions produced during reperfusion were greater than those produced during ischemia, in mesenteric I/R in felines. I/R can provoke complex interactions between the endothelium and different cell types, leading to microvascular injury, cellular necrosis and/or apoptosis (Massberg & Messmer, 1998). Ischemia and reperfusion of the small intestine provoke the rupture of the mucosa barrier, bacterial translocation and the activation of inflammatory responses (João *et al.*, 2004). Histological damage that occurs after intestinal I/R in the form of shortening of the villi length, loss of villus epithelium, dilatation and congestion of blood vessels, degeneration of epithelial cells with nuclear alterations and invasion by inflammatory cells were coincided with the results of the present work (Tunc *et al.*, 2009). In *Potentilla fulgens* group, slightly breaking the apical and lateral surfaces of the villi, connective tissue beneath the epithelium were observed in a small number of inflammatory cell infiltration. Villi in the intestine fully protected, significant damage are not observed in the mucosa (Fig. 1C).

Immunohistochemistry with PCNA antibody and the TUNEL method have been widely used in fish species to demonstrate the renewal of enterocytes in studies ranging from experimental diet to environmental and toxicological assessments (Ferrando *et al.*, 2005; Pohlenz *et al.*, 2012).

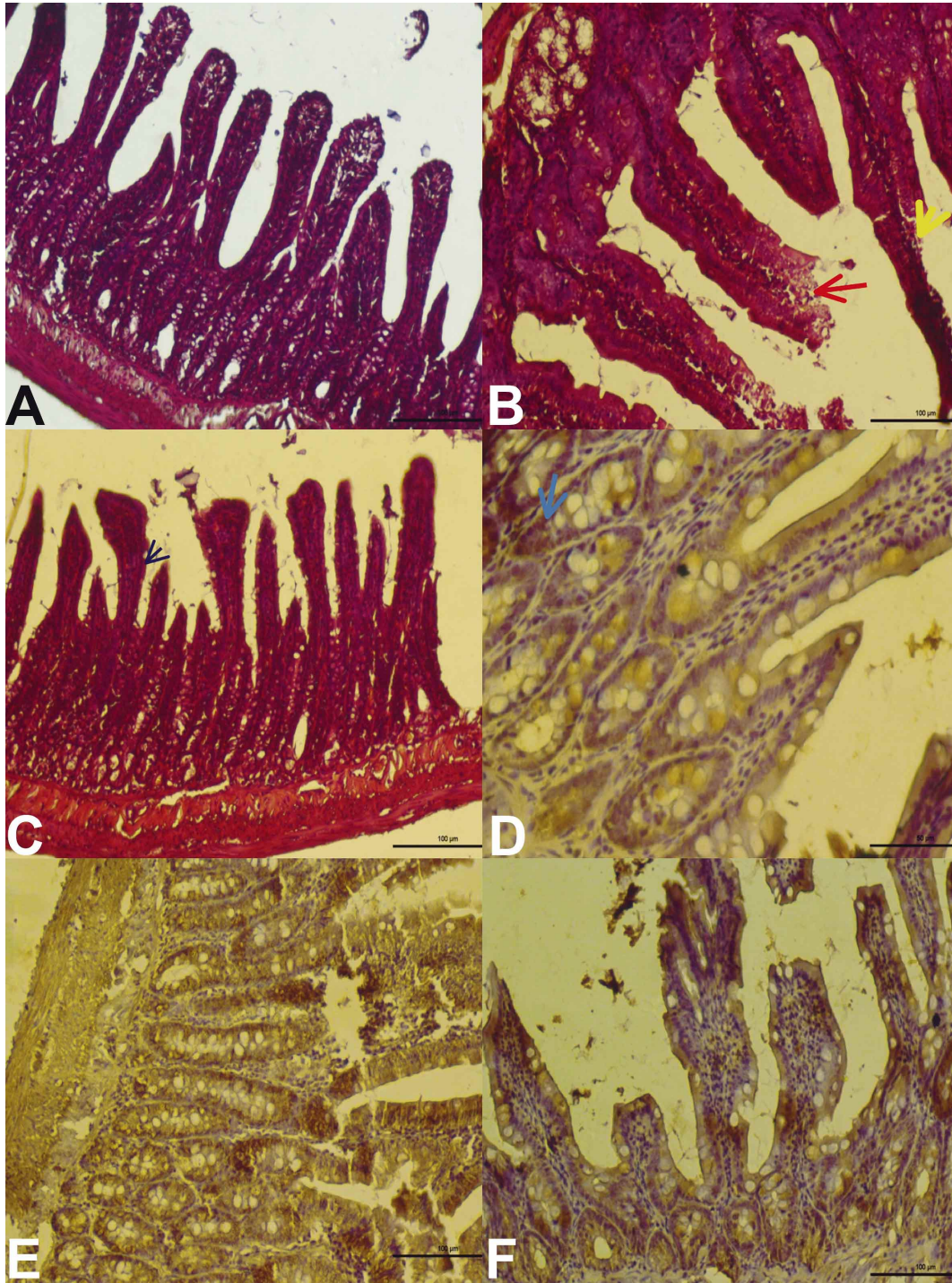


Fig. 1. A) Sham group, Normal appearance in intestinal mucosa (H-E staining, Bar 100 µm). B) Decrease and broken in villi structure (yellow arrow), edema and necrosis in subepithelial lamina propria (red arrow) (H-E staining, Bar 50 µm). C) Slight break in the apical and lateral surfaces of the villi, (arrow) a small number of inflammatory cell infiltration in the connective tissue beneath the epithelium (H-E staining, Bar 100 µm). D) Control group, PCNA positive nuclei in the crypt and glands region (PCNA immunostaining and hematoxylin stain, Bar 50 µm). E) Ischemia-reperfusion group the breakage of the villi and crypts in long, increased PCNA expression (PCNA immunostaining and hematoxylin stain, Bar 100 µm). F) Ischemia-reperfusion+*Potentilla fulgens* group, Intestinal epithelium and crypts in the regular distribution of positive cells (PCNA immunostaining and hematoxylin stain, Bar 100 µm).

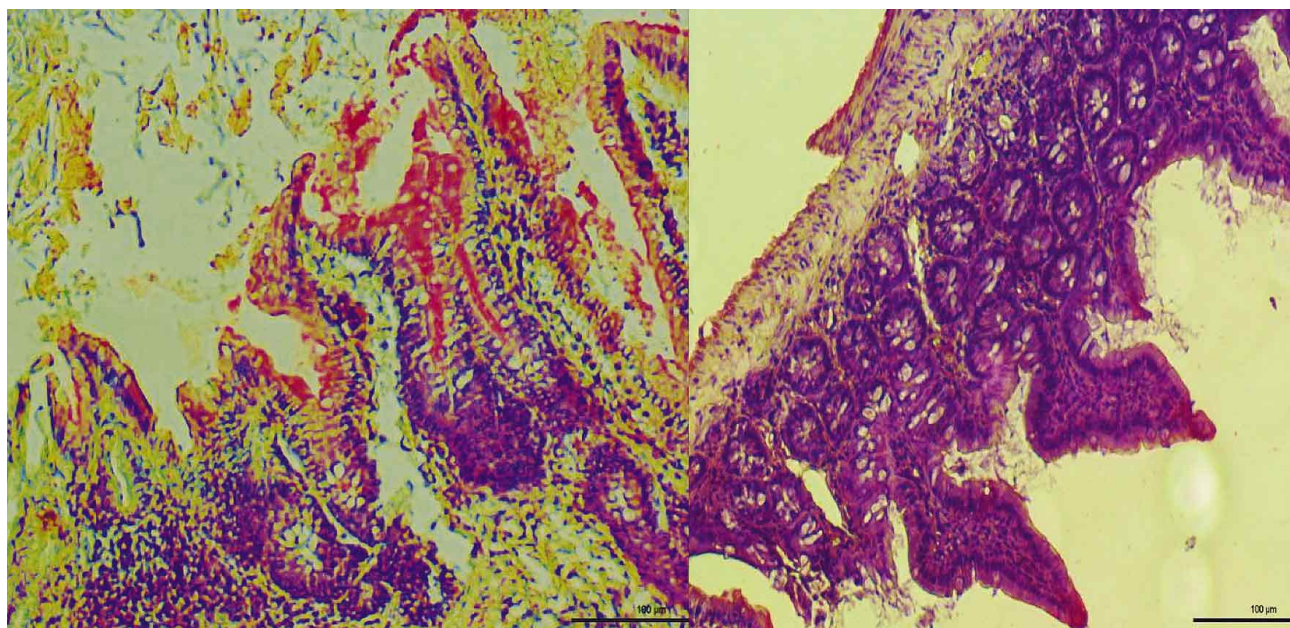


Fig. 2. A) Ischemia-reperfusion group A increase of apoptotic cells in intestinal epithelium and deep crypts (Tunnel method (PCNA), Hematoxylin stain, Bar 50 µm). B) Ischemia-reperfusion+*Potentilla fulgens* group A small number of apoptotic cells in the surface epithelium (Tunnel method (PCNA), Hematoxylin stain, Bar 50 µm).

Table III. The data of MDA levels of control and experimental groups of rats.

Indices (Rat) n= 10	Ischemi-reperfusion group	Ischemi-reperfusion + <i>PotentillaFulgens</i> group
1	48	21
2	46	19
3	38	24
4	49	19
5	50	20
6	52	23
7	39	22
8	40	21
9	49	20
10	36	22
Mean	44.7±5.6	20.3±1.6

This is in accordance with result of some investigators who reported non significant increase in the number of PCNA-positive cells in the section obtained after 240 min from reperfusion (Shima *et al.*, 2006). Our study demonstrated that the expressing levels of PCNA after reperfusion were higher than the levels of control in both crypt and villus epithelial cells. These findings indicated that the repair process of small intestinal mucosa was

initiated by I/R and cellular regeneration continued for following reperfusion. A recent study also tested the acute toxicity of *P. fulgens* root extract and did not find any mortality or symptoms of toxicity in animals up to 4000 mg/kg (Laloo *et al.*).

Laloo *et al.*, have also reported that 200 and 400 mg/kg, p.o. dose of *P. fulgens* root ethanolic extract significantly inhibits ethanol and pyloric ligation-induced gastric ulcers due to its anti-secretory properties.

The small intestine is highly sensitive to ischemia-reperfusion. It has been demonstrated that occlusion of the superior mesenteric artery followed by reperfusion can cause apoptosis in the intestinal epithelium (Noda *et al.*, 1998). The improvement in the intestinal mucosa of *Potentilla fulgens* group, that we have a hyperplastic intestinal mucosa with a high proliferation of supported to increase the absorptive surface of adaptive responses. These results suggest that *Potentilla fulgens* treatment could inhibit cellular apoptosis of mucosal cells and induced cell proliferation to accelerate regeneration and repair of small intestinal mucosa after ischemia-reperfusion insult. *Potentilla fulgens* exerts a positive protective effect on the mucosal barrier and decreases the intestinal permeability. Its administration may be helpful in patients with a risk of intestinal ischemic disease.

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RESUMEN: El objetivo fue investigar los efectos protectores de *Potentilla fulgens* sobre la lesión por isquemia-reperusión en ratas albinas Wistar (n= 30) con un peso de 180 g. En el grupo 1, los animales fueron sometidos a laparotomía simulada sin lesión por isquemia-reperusión. En el Grupo 2, los animales fueron sometidos a laparotomía y oclusión de la arteria mesentérica superior durante 30 min seguido de 20 min de reperusión sin pretratamiento. El grupo *Potentilla fulgens* recibió 400 mg/kg/día de *P. fulgens* por vía intraperitoneal 5 días antes de la lesión por isquemia-reperusión. Hubo diferencias significativas entre el grupo de grupo con isquemia-reperusión y el tratado con *Potentilla fulgens* (p<0,0001). En el análisis estadístico del nivel de malondialdehído (MDA), los datos se obtuvieron después de una medición respectiva en todos los grupos. Los grupos *Potentilla fulgens* y con isquemia-reperusión tuvieron una disminución significativa de MDA (p<0,0001). En el periodo después de la isquemia-reperusión, se observó inmunoreactividad del marcador PCNA en los núcleos de las células de las criptas y vellosidades. En el grupo de isquemia-reperusión, la inmunoreactividad a PCNA fue bastante avanzada y se extendió a lo largo de la parte media de los pliegues intestinales. También aumentó el número de núcleos positivos a TUNEL. En el grupo isquemia-reperusión tratado con *P. fulgens*, el epitelio intestinal mostró pocos núcleos inmunoreactivos a PCNA; núcleos positivos a TUNEL se observaron en el lumen intestinal y la mucosa, cerca de las células calciformes diferenciadas. Demostramos que el extracto de *P. fulgens* disminuye significativamente lesiones de la mucosa intestinal causadas por la isquemia-reperusión

PALABRAS CLAVE: Isquemia intestinal; Antígeno nuclear de proliferación celular (PCNA); Rata; Método de tunel.

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