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Graptophyllum pictum Extract in the Treatment of Experimental Hemorrhoids: Effects on Vascular Leakage and Matrix Metalloproteinase-9 Levels

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Abstract

BACKGROUND: The micronized purified flavonoid fraction, which has been shown to be effective for the treatment of hemorrhoids, is too expensive for Indonesian patients and is not included in the National Formulary.

AIM: The aim of this study was to investigate the effects of Graptophyllum pictum extract (GPE), a phlebotropic drug, as it is a cheaper and safer alternative medication for the treatment of hemorrhoids.

MATERIALS AND METHODS: Twenty-eight male Wistar rats were randomly divided into four groups. Hemorrhoids were induced in groups 2, 3, and 4 using 6% croton oil. After induction, group 1 (negative control) and group 2 (positive control) were administered normal saline, whereas groups 3 and 4 were administered 100 mg/kg BW and 300 mg/kg BW GPE, respectively. On the 9th day, blood samples were collected to measure serum matrix metalloproteinase (MMP)-9 levels. The anus, containing the internal and external sphincters, was resected. Vascular leakage was measured based on edema and extravascular leukocyte count. The edema was measured using the rectoanal coefficient.

RESULTS: The highest rectoanal coefficient was observed in groups 2 (3.13 ± 0.85) and 3 (2.46 ± 0.41); that in group 4 (2.60 ± 0.34) was significantly lower than that in group 2 ($p < 0.05$). The highest leukocyte count was observed in groups 2 (1003.28 ± 99.30) and 3 (900.14 ± 48.09); that in group 4 (835.85 ± 42.65) was significantly lower than that in group 2 ($p < 0.05$). The highest mean serum MMP-9 level was observed in groups 2 (1840.25 ±437.84) and 3 (525.78 ± 577.33); that in group 4 (1122.03±675.76) was significantly lower than that in group 2 ($p < 0.05$).

CONCLUSIONS: GPE effectively reduced vascular leakage (edema and extravascular leukocyte count) and MMP-9 level in this experimental model of hemorrhoids.

Introduction

A hemorrhoid is the prolapse of the normal anal cushion [1]. An epidemiological study in 1990 reported that more than 10 million Americans suffer from hemorrhoids, with a prevalence of 4.4%. The highest prevalence was detected in the age range of 45–65 years in both sexes [2], [3]. In the United Kingdom, in 2005, it was reported that hemorrhoids are common, affecting one in every four people, and more than 20,000 hemorrhoidal procedures are carried out every year [4]. In Indonesia, data from the Ministry of Health show that, in 2015, the prevalence of hemorrhoids was 5.7% or approximately 12.5 million people [5].

In general, hemorrhoids are divided into three types: Internal, external, and mixed [4], [6]. The histopathological changes in the anal cushion of a hemorrhoid specimen are the dilatation of the vein, vascular thrombosis, the degeneration of collagen and fibroelastic tissue, and the distortion or deterioration of subepithelial muscles. In addition, inflammation occurs in the vascular wall and the surrounding tissues, which causes ulcers, ischemia, and thrombosis [1].

The pathophysiology of hemorrhoids includes chronic straining, which damages or weakens the muscularis mucosa, muscle of Treitz, and ligament of Parks. The damage to these structures causes the prolapse of the anal cushion. These prolapses are strangulated by the internal sphincter of the anus, which blocks venous return and causes thrombosis [1], [5], [7]. The blocked venous return causes venous hypertension, which leads to the activation of leukocytes, macrophages, and endothelial cells. These inflammatory cells degrade the extracellular matrix through the release of oxygen free radicals and matrix metalloproteinase (MMP) [1].

The main complaint of hemorrhoids is the bleeding of fresh red blood from the anus, accompanied by a protrusion from the anus, itching, and pain [6]. The internal hemorrhoid is divided into

four degrees, and its management is based on the degree. The 1st and 2nd degrees of hemorrhoids are managed non-operatively, with a high fiber diet, medication, and, if necessary, office-based treatment, such as sclerotherapy, rubber band ligation, and infra-red coagulation [1], [2]. The 3rd and 4th degrees of hemorrhoids are managed operatively. The medications used are anti-inflammatory and phlebotropics [1], [3]. A phlebotropic drug, now available in Indonesia, is a micronized purified flavonoid fraction (MPFF) that contains a combination of diosmin and hesperidin. In a meta-analysis of RCTs, MPFF has been shown to suppress hemorrhage and pain as a non-operative hemorrhoid [8] and post-hemorrhoidectomy [9] medication. However, in Indonesia, MPFF is imported, considered expensive, and not included in the National Formularies; therefore, it cannot be prescribed to patients covered by national insurance. Instead, cheaper local products that act as phlebotropics for the treatment of hemorrhoids are needed. Several anti-hemorrhoid herbal medications are offered in Indonesia, among which, some are registered by the "Badan Pengawas Obat dan Makanan" (National Agency of Food and Drug Control) and contain *Graptophyllum pictum* extract (GPE), also known as "Purple Leaf" or "Daun Ungu." [10]. In experimental rats with inflammation induced on their feet, GPE acts as an anti-inflammatory agent with comparable efficacy to indomethacin [11]. However, no studies have investigated the anti-inflammatory effects of GPE as a phlebotropic on hemorrhoids. In this study, the effects of GPE as a phlebotropic medication for the treatment of hemorrhoids were investigated.

Materials and Methods

This was a randomized controlled trial with a post-test design to evaluate the effects of GPE as a phlebotropic by measuring vascular leakage (using anus weight and rectoanal coefficient, which represent edema and extravascular leukocyte count) and the expression MMP-9 in hemorrhoid-induced Wistar rats. Croton oil, which has already been used in the previous studies, was used to induce hemorrhoids in the Wistar rat anus.

Animals

The subjects were male Wistar rats (age, 10–12 weeks; weight, 200 g) provided by the "Laboratorium Penelitian dan Pengujian Terpadu" (LPPT), Gajah Mada University. The experiment and enzyme-linked immunosorbent assay (ELISA) measurements were conducted at the LPPT, Gajah Mada University, whereas extravascular leukocyte counts from anal specimens were conducted at the Pathology Anatomy Laboratory, Faculty of Medicine, Diponegoro University from October 2019 to January 2020. Each sample group consisted of seven rats. The animals were provided with a standard chow diet and housed with their group at room temperature (20°C). The animals were treated in accordance with the guide for the care and use of laboratory animals.

Croton oil consisted of deionized water, pyridine, diethyl ether, and croton oil (6%) in diethyl ether at 1:4:5:10. The rats were fasted overnight before the start of the experiment. A 4-mm cotton applicator dipped in 100 μL of croton oil was dabbed on the rectoanal area, up to 15 mm from the anal verge, for 30 s. Induction was performed on 3 consecutive days[12]. The GPE was prepared by the Sido Muncul Herbal Medicine Factory. The purple leaves, harvested from the Sido Muncul Herbal Farm, were dried and processed to form a powder. The powder was extracted using 70% ethanol using a previously described method.

Procedure

The 28 male Wistar rats were randomly allocated to four different groups. After random allocation, the weight of the Wistar rats was measured. Hemorrhoids were not induced in group 1 (negative control), whereas the other three groups were exposed to 6% croton oil on the anal canal for 3 days to induce hemorrhoids.

After induction with croton oil for 3 days, Wistar rats were treated as follows for 5 consecutive days: Groups 1 (negative control) and 2 (positive control) were administered normal saline, group 3 was administered GPE at 100 mg/kg BW, and group 4 was administered GPE at 300 mg/kg BW. On the 9th day, the rats were weighed (in g), blood samples were extracted from the retro-orbital plexus to measure serum MMP-9 levels, and the rats were euthanized. Serum MMP-9 levels were measured using ELISAs. The anus, containing the internal and external sphincter, was resected, with a 2-cm proximal margin from the outer border of the anus. The specimens were weighed (in mg) and prepared for histological examination. The rectoanal coefficient was measured as the ratio of anal weight (mg) to body weight (g) [11]. Hematoxylin and eosin staining were used and extravascular leukocytes were counted in 400 high-power fields.

Statistical analysis

The rat weight, anal weight, rectoanal coefficient, MMP-9 level, and extravascular leukocyte count were normally distributed; therefore, the differences among and between groups were analyzed using ANOVA and least significant difference (LSD). Statistical significance was set at $p < 0.05$.

Results

All rats were still alive and actively mobile at the end of the study; therefore, statistical analyses were performed based on the numbers of rats at random allocation. The mean (±standard deviation [SD]) weights (g) of Wistar rats before manipulation in groups 1, 2, 3, and 4 were 192.61 (±22.40), 161.88 (±5.19), 166.61 (±11.69), and 171.60 (±12.55), respectively, (ANOVA; p = 0.003). As anal weight may correlate with body weight, measuring the rectoanal coefficient (ratio of anal weight to body weight) will provide more accurate information regarding the degree of edema. The LSDs of Wistar rats before euthanasia in groups 1, 2, 3, and 4 were 219.61 (±26.57), 173.84 (±13.3), 177.62 (±14.5), and 171.70 (±13.1), respectively, (ANOVA; p = 0.000). The LSDs of group 1 versus 2, group 1 versus 3, group 1 versus 4, group 2 versus 3, group 2 versus 4, and group 3 versus 4 were p = 0.000, p = 0.000, p = 0.000, p = 0.695, p = 0.824, and p = 0.540, respectively, (Figure 1).

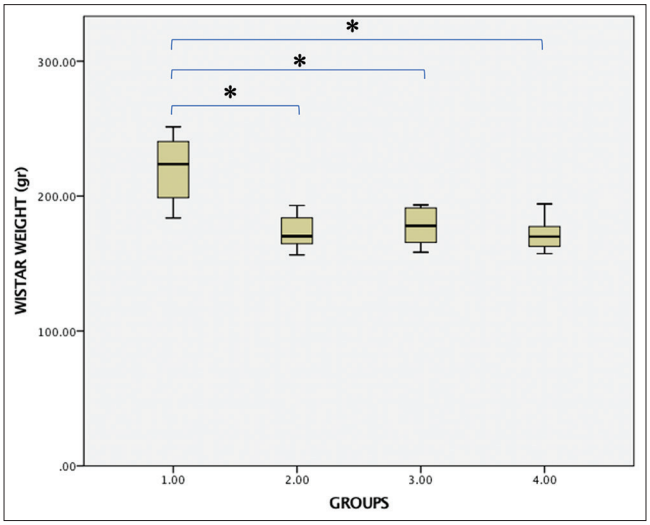

*Figure 1: Mean (±SD) weight (g) of Wistar rats before euthanasia in groups 1 (negative control), 2 (positive control), 3 (GPE 100 mg/kg BW), and 4 (GPE 300 mg/kg BW). ANOVA, p = 0.000; LSD, * p < 0.05*

The mean (±SD) anus weights (mg) of Wistar rats in groups 1, 2, 3, and 4 were 407.84 (±84.97), 551.87 (±96.99), 431.68 (±64.39), and 446.08 (±63.62), respectively, (ANOVA; p = 0.011). The LSDs of group 1 versus 2, group 1 versus 3, group 1 versus 4, group 2 versus 3, group 2 versus 4, and group 3 versus 4 were p = 0.002, p = 0.577, p = 0.373, p = 0.009, p = 0.019, and p = 0.735, respectively, (Figure 2).

The mean (±SD) rectoanal coefficients in groups 1, 2, 3, and 4 were 1.88 (±0.52), 3.13 (±0.85), 2.46 (±0.41), and 2.60 (±0.34), respectively, (ANOVA; p = 0.004). The LSDs of group 1 versus 2, group 1 versus 3, group 1 versus 4, group 2 versus 3, group 2 versus 4, and group 3 versus 4 were p = 0.000, p = 0.071, p = 0.027, p = 0.036, p = 0.091, and p = 0.648, respectively, (Figure 3).

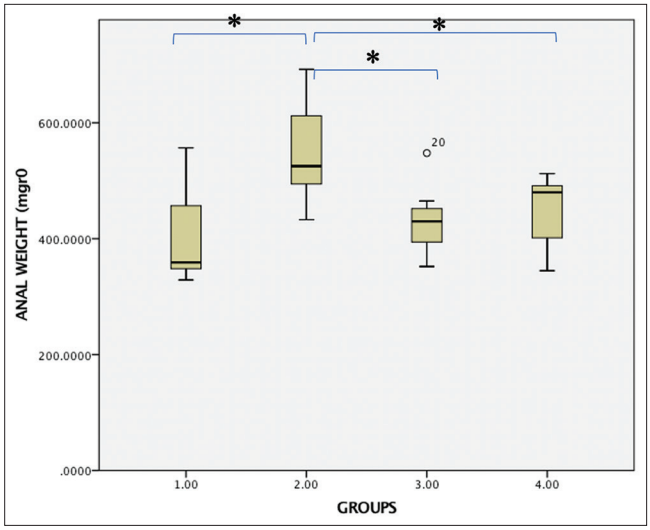

*Figure 2: Mean (±SD) anus weight (mg) of Wistar rats in groups 1 (negative control), 2 (positive control), 3 (GPE 100 mg/kg BW), and 4 (GPE 300 mg/kg BW). ANOVA, p = 0.011; LSD, * p < 0.05*

The mean (±SD) extravascular leukocyte counts of the anal specimen in groups 1, 2, 3, and 4 were 862.28 (±138.64), 1003.28 (±99.30), 900.14 (±48.09), and 835.85 (±42.65), respectively, (ANOVA; p = 0.012). The LSDs of group 1 vs. 2, group 1 vs. 3, group 1 vs. 4, group 2 vs. 3, group 2 vs. 4, and group 3 vs. 4 were p = 0.000, p = 0.203, p = 0.296, p = 0.009, *P* = 0.000, and p = 0.026, respectively, (Figure 4).

The mean (±SD) serum MMP-9 levels in groups 1, 2, 3, and 4 were 935.21 (±103.24), 1840.25 (±437.84), 525.78 (±577.33), and 1122.03 (±675.76), respectively, (ANOVA; p = 0.001). The LSDs of group 1 versus 2, group 1 versus 3, group 1 versus 4, group 2 versus 3, group 2 versus 4, and group 3 versus 4 were p = 0.002, p = 0.137, p = 0.490, p = 0.000, p = 0.13, and p = 0.035, respectively, (Figure 5).

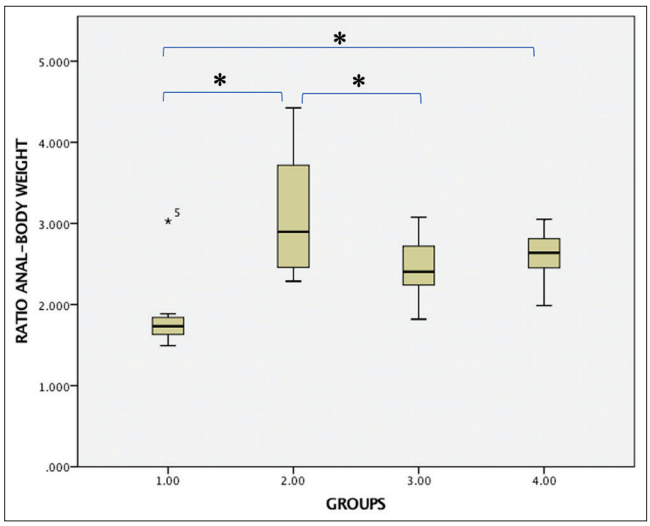

*Figure 3: Mean (±SD) rectoanal coefficient in groups 1 (negative control), 2 (positive control), 3 (GPE 100 mg/kg BW), and 4 (GPE 300 mg/kg BW). ANOVA, p = 0.004; LSD, * p < 0.05*

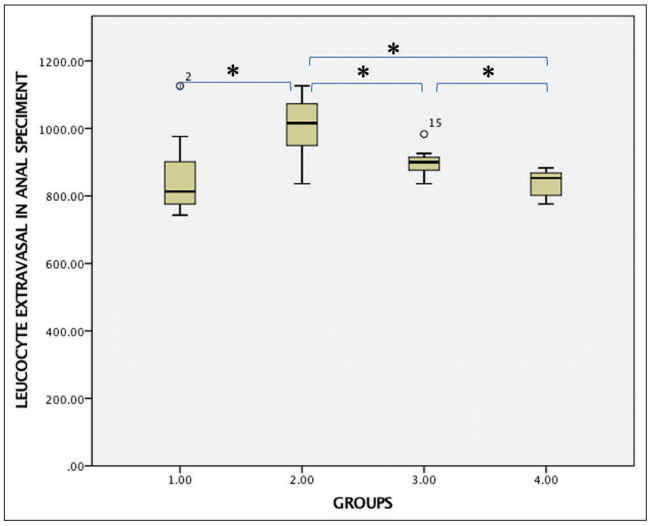

*Figure 4: Mean (±SD) extravascular leukocyte count in anal specimen in groups 1 (negative control), 2 (positive control), 3 (GP 100 mg/kg BW), and 4 (GP 300 mg/kg BW). ANOVA, p = 0.012; LSD, * p < 0.05*

Discussion

The previous studies have reported that the application of 6% croton oil results in anal inflammation and, pathologically, the hemorrhoids induced show not only vascular dilatation but also inflammation [1]. Therefore, 6% croton oil is considered a standard for experimental hemorrhoid production. Croton oil may increase proinflammatory cytokine expression, vasodilatation, capillary permeability, and vascular leakage. Vascular leakage results in edema and an increase in the level of macrophages in the anal parenchyma [5], [6]. Edema increases the anal weight. This study showed that after the application of 6% croton oil (by comparing the negative control and positive control groups), the rectoanal coefficient, extravascular leukocyte count, and MMP-9 levels were significantly increased, indicating that croton oil induced inflammation. As the purpose of this study was to determine the efficacy of GPE, the reduction in vascular leakage (edema and extravascular leukocyte count) and MMP-9 levels was compared among anal specimens exposed to 100 mg GPE/kg BW, 300 mg GPE/kg BW, and a positive control.

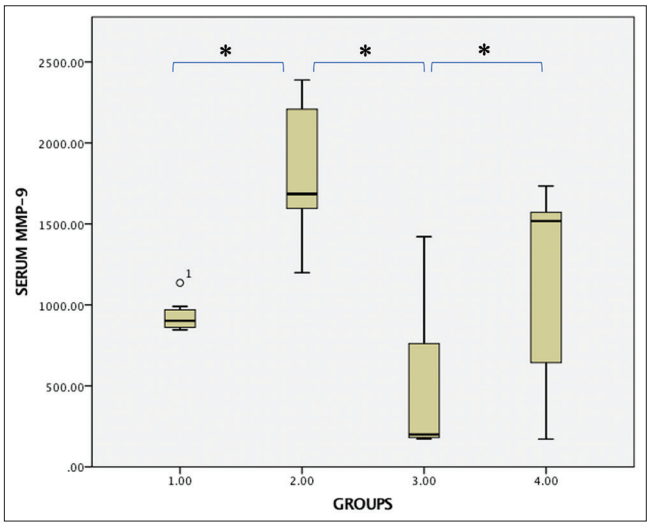

*Figure 5: Mean (±SD) serum MMP-9 level in groups 1 (negative control), 2 (positive control), 3 (GP 100 mg/kg BW), and 4 (GP 300 mg/kg BW). ANOVA, p = 0.001; LSD, * p < 0.05*

This study showed that the weight of anal specimens in 100 and 300 mg GPE/kg BW groups was significantly lower than that in the positive control group. The rectoanal coefficient in the 100 mg GPE/kg BW group was significantly lower than that in the positive control group; that in the 300 mg GPE/kg BW group was also lower than that in the positive control group, but the difference was not statistically significant. These results show that GPE, especially at a dose of 100 mg/kg BW, effectively reduces the anal edema induced by croton oil.

In acute inflammation, neutrophils move outside the vascular lumen due to the increased vascular permeability. Neutrophils eradicate bacteria or necrotic tissues that stimulate the inflammatory reaction. During the healing process, the levels of neutrophils are decreased and they are replaced by macrophages[10], [11]. In this study, the number of leukocytes outside the vascular lumen was significantly lower in the GPE group than in the control group. A GPE dose of 300 mg/kg BW resulted in the lowest extravascular leukocyte count. This result indicated that GPE reduces leukocyte leakage outside the vascular lumen. As GPE reduces edema and the number of leukocytes outside the vascular lumen, it can be concluded that GPE reduces vascular leakage and increases the healing process.

The extracellular matrix (ECM), a non-cellular component that is present in all tissues and organs, is essential for the physical scaffolding of cellular constituents. Fundamentally, the ECM is composed of water, proteins, and polysaccharides. Cell adhesion to the ECM is mediated by several ECM receptors, such as integrins, discoidin domain receptors, and syndecans. Collagen type I–III, elastin, fibronectin, tenascin, and a repertoire of proteoglycans (e.g., hyaluronic acid and decorin) produced by non-activated tissue fibroblasts, maintain the structural and functional integrity of the interstitial ECM. In hemorrhoids, the degradation of the ECM is suspected to be the cause of the prolapse of the anal cushion. The studies have shown that in hemorrhoids, the collagen-protein[7] and collagen type I–III ratios are significantly lower than those in normal anal cushion. Collagen type I fibers have immense tensile strength and can withstand enormous forces, whereas type III collagen fibers are thinner and more immature; therefore, reducing the ratio of collagen I-III reduces the mechanical stability of connective tissue [8]. MMPs are enzymes that regulate the ECM. MMP-2 (gelatinase A) and MMP-9 (gelatinase

B) readily digest denatured collagens and gelatins [11]. MMP-1, -2, -8, -9, and -14 can cleave collagen, and MMP-9 plays a major role in the degradation of the ECM in a range of physiological and pathophysiological processes that involve tissue remodeling. MMP-9 is also reported to play a significant role in neovascularization through the proteolytic degradation of proteins in the basal lamina of blood vessels and release of the biologically active form of vascular endothelial growth factor. MMP-9 is secreted by a number of cell types, including neutrophils, macrophages, and fibroblasts, and is overexpressed during acute inflammation [11]. Moreover, compared with that in normal anal tissues, the expression of MMP-2, -3, -7, and -9 is significantly increased in grade III hemorrhoidal tissues, and that of MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL) are significantly increased in grade IV hemorrhoidal tissues. NGAL modulates the activity of MMP-9 in vascular disease. In this study, the serum MMP-9 levels of the experimental hemorrhoids were significantly lower in 100 and 300 mg GPE/kg BW groups than in the control group. This result can be explained by the inflammatory effects of GPE [11]. In this study, extravascular leukocyte infiltration was lower in the GPE group than in the control group, and MMP-9 was overexpressed.

Conclusions

GPE extract, especially at a dose of 100 mg/kg BW, decreased vascular leakage and MMP-9 levels in Wistar rats with experimental hemorrhoids. As GPE is already used as a traditional medicine among Indonesian people, it has proven to be safe, further clinical research should be carried out.

Acknowledgments

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