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Histomorphometric study of ethanolic extract of *Graptophyllum pictum* (L.) Griff. leaves on croton oil-induced hemorrhoid mice: A Javanese traditional anti-hemorrhoid herb

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ABSTRACT

Ethnopharmacology relevance: *Graptophyllum pictum* (L.) Griff., known as "handeuleum" in West Java and "Daun Ungu" in Indonesia, is traditionally used to cure hemorrhoids.
Aim of the study: The purpose of this study is to prove its effectiveness scientifically using anorectal histological parameters in Croton oil-induced hemorrhoid mice.
Materials and methods: In vivo tests were performed by observing histomorphologic changes in mice anorectal tissue induced by croton oil. In addition, in vitro assay was performed for evaluating antioxidant activity, astringency property, and hemostasis-associated activity. The antioxidant activity was measured using a DPPH radical scavenging assay. The total flavonoid and phenolic contents were also determined spectrophotometrically.
Results: The in vivo assay showed that the oral-topical combination use of the ethanolic extract of *G. pictum* leaves demonstrated significant improvement on the croton oil-induced anorectal damage better than the single application by oral or topical application.
Conclusion: These results showed that *G. pictum* has potent anti hemorrhoid activity, especially for the combinational use of oral and topical administration.

1. Introduction

Hemorrhoid is a common disease from which humans have suffered through the ages. Chronic progression of anorectal ailment is caused by various factors that require intensive medical intervention and may cause socio-economical loss. Until now, patients continue to seek better healing treatment, both surgical and non-surgical methods to balance patient satisfaction, postoperative complications, pain, and relapse rate (Čuk et al., 2015).

One of the features of hemorrhoids is swollen veins in the rectum or anus. Internal hemorrhoid that involves the veins far inside of rectum do not hurt because of a lack of pain-sensing nerves but often causes bleeding and the urge to defecate. The recurrence of internal hemorrhoids increases the size and swells out from the rectum, which induces itch and pain. External hemorrhoids involving a vein outside of the anus, where sufficient pain-sensing nerves exist, cause itch and severe pain (Mounsey et al., 2011; Sun and Migaly, 2016; Zaman et al., 2015).

Hemorrhoids are caused by the disintegration or alteration in the anal cushion support tissue such as abnormal venous dilatation, vascular thrombosis, degeneration of collagen fibers and fibroelastic tissue,

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distortion, and rupture of the rectal sub-epithelial muscle. It is also found severe inflammation in blood vessel walls and connective tissue (Loder et al., 1994; Lohsiriwat, 2012; Sardiñas et al., 2016; Sun and Migaly, 2016).

Various nutritional therapies and plant extracts for hemorrhoids had been carried out and showed significant results even though scientific evidence was still lacking (Mackay, 2001). Some plant extracts containing anti-inflammatory and antioxidative constituents have the potential for hemorrhoidal therapy and have also been shown to have increased vascular tone, capillary flow, strengthening connective tissue, and perivascular microcirculation. These plant extracts are used in various forms, both orally and topically, but only a few plants have been studied scientifically (Yildirim et al., 2017).

A variety of hemorrhoid therapy using both oral and topical medications has been known until now in traditional or modern ways. In general, these drugs are intended to relieve hemorrhoid symptoms, but unfortunately, they are unsuccessful in many cases. The combination of oral and topical medications aims to increase the effectiveness of hemorrhoid treatment, avoiding invasive surgery (Misra and Imlitemsu, 2005).

Graptophyllum pictum (L.) Griff. is a species of Acanthaceae family known as "handeuleum" in West Java and "Daun Ungu" in Indonesia (Levang and Foresta, 1991; Ramdhan et al., 2015). *G. pictum* leaves (GPL) have historically been used to treat hemorrhoids (Heyne, 1987; Ministry of Health, 2010). The determination of analgesic and anti-inflammatory capabilities (Ozaki et al., 1989), the analysis of phagocytosis behavior, and immunoglobulin formation (Kusumawati et al., 2002) and the activity on the classical pathway of complement and chemoattractant activity (Kusumawati et al., 1997) are just some of the pharmacological activities relevant to hemorrhoid. However, further scientific evidence is still required to prove its effectiveness for future development as an anti hemorrhoid remedy.

The chemical content of GPL has been briefly reported in several studies. GPL contains essential oils such as phytol (75.7%), n-nonacosane (6.5%) and hexahydrofarnesyl acetone (2.6%) (Jiangseubchatveera et al., 2015) and other chemical substances such as myricetin and kaempferol (Kusumawati et al., 2002), alkaloid, glycoside, steroid, saponin, tannin, calcium oxalate (Ministry of Health, 2010). Ozaki suggests that the flavonoid compounds in GPL play a role in anti-inflammatory activity (Ozaki et al., 1989). Flavonoids from Ginkgo biloba have been shown to increase venous tone and lymphatic drainage, decreasing capillary hyperpermeability from inflammatory processes, and have been successfully used in clinical trials in hemorrhoidal patients (Misra and Imlitemsu, 2005; Zaman et al., 2015).

The purpose of this study is to determine the histological activity of ethanol extract of GPL (GPLE) given orally, topically, and combination (orally-topically) in croton oil-induced hemorrhoid mice. Hemorrhoid symptoms are bleeding, itching, and pain due to hard stools (Yamana, 2017), so the astringency properties and hemostasis-associated activity of GPLE were also discussed.

2. Materials and methods

2.1. Drugs and chemical

Folin-Ciocalteu and DPPH reagents were obtained from Sigma Co. chemicals. Betamethasone was purchased from PT. Kimia Farma, Indonesia. All other chemicals are the highest purity and analytical grade.

2.2. Plant material and extract preparation

The GPL was obtained from a farm in Lawang tea plantation, Malang, East Java, Indonesia, in October 2017. A voucher specimen (RM GP102017) was identified and deposited in the Herbarium of the Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Airlangga University. The dried GPL was grounded into a powder. The powder (100 g) was extracted using 70% ethanol (plant: solvent, 1:10, w/v), in a microwave (30% generator power), for 1 min. The extracts were dried by evaporating the solvent under reduced pressure then freeze-dried.

2.3. Determination of total flavonoid content

According to Christ and Müller's method (Christ and Muller, 1960), the aglycone of flavonoid was released by acid hydrolysis of the GPLE, and then reacted with $AlCl_3$ in a methanol-ethyl acetate-acetic acid solvent to form a complex. The specific absorbance of the complex was measured using a spectrophotometer at 425 nm. The experiments were conducted in quintuplicate, and the amount was expressed as hyperoside equivalent (HE, mg/100 g samples) (Jafari et al., 2010; Nan et al., 2012).

2.4. Determination of total phenolic content

The measurement of total phenolic contents in GPLE was performed using a spectrophotometer as gallic acid equivalents using the Folin-Ciocalteu reagent according to the standard method (Singleton and Rossi, 1965). Gallic acid (10–500 mg/L) was used for a standard calibration. Folin-Ciocalteu solution (1:10 v/v in water) and sodium bicarbonate solution (7.5% w/v) were used as reagents. Each sample and standard (40 μL) was mixed with 1.8 mL of Folin-Ciocalteu reagent for 5 min at room temperature, and then added 1.2 mL of sodium bicarbonate. After 60 min, the absorbances were measured at 765 nm. The results were expressed as gallic acid equivalent (GAE, mg/100 g samples) (Odeh et al., 2014).

2.5. DPPH radical scavenging activity

The antioxidant activity was evaluated based on DPPH radical scavenging activity assay (Kusumawati et al., 2018; Kusumawati and Indrayanto, 2013). The GPLE solution was mixed with 100 μL freshly prepared DPPH methanolic solution (250 mM) on a 96-well microplate in triplicate. After incubation in the dark for 30 min, the remaining DPPH radical was evaluated from the absorbance at 515 nm, using a Multiscan Go Thermo Scientific microplate reader. DMSO was used as a negative control and Trolox as a positive control. The IC_{50} of the inhibition ratio was determined by linear regression.

2.6. Measurement of astringent properties

Astringent activity-induced vasoconstriction was an essential factor in hemostasis (Nabavizadeh et al., 2016). Astringency activity was determined using the milk precipitation method (Dandjesso et al., 2012; Klotoé et al., 2012). Briefly, 1 mL of GPLE solution (5%) was put in the tube, added with 100 μL milk and homogenized, allowed to stand for 3 min, and centrifuged for 1 min at 3000 rpm. The formation of a pellet was observed as the astringent activity.

2.7. Measurement of plasma recalcification time

Plasma recalcification time (PRT) was measured to determined sample-induced effect in the clotting time of Plasma Poor Platelet (PPP) following activation of prothrombin (Factor II) by the addition of Ca^{2+} (Elahi et al., 2014). In this method, 0.1 mL PPP (defrosted and incubated at 37 °C) and 0.1 mL of the different sample solutions were combined well in test tubes (8 mm diameter). After incubation for 5 min in a 37 °C water bath, 0.1 mL $CaCl_2$ solution (0.025 mM) was added to each tube. The time necessary for silky fibrin formation was recorded as the PRT. Saline was used as a negative control and tranexamic acid as a positive control.

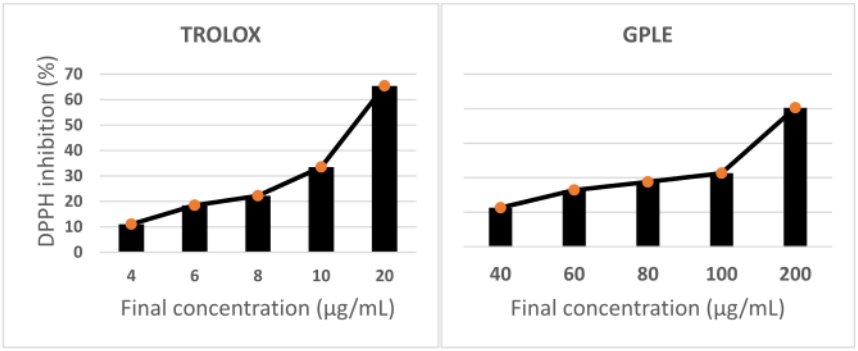

Fig. 1. Dose-dependent antioxidant activity of GPLE. (n = 3).

2.8. Animals and histomorphologic analysis of croton oil-induced hemorrhoid mice

Male mice (ICR), aged three months and weighing 20–25 g, were used in this study. They were obtained from the laboratory of the animal center, Faculty of Pharmacy, Airlangga University. Animal experiments were designed based on the ethical standards for animal use and were approved by the Airlangga University Ethical Committee of Animal Experimentation (protocol number 2.KE.88.05.2018). For the oral group, GPLE suspension was given to mice once daily with oral gavage at the same time without fasting. The dosage volume was set at 10 mL/kg of the body weight. For the topical group, the GPLE gel was set at 250 mg/kg and applied intrarectally on mice once daily at the same time. All samples were applied for 14 days.

2.8.1. Experimental design

Experiments were carried out using croton oil-induced hemorrhoid mice model based on the modified method described by Nishiki et al. (1988) and Azeemuddin et al. (2014). Mice were randomly divided into eight groups (8 animals each). The normal (N) group was healthy animals. For the other groups, hemorrhoid was induced by rubbing a sterile cotton swab into the anorectal for 10 s once a day for five days with a mixture of croton oil (deionized water, pyridine, diethyl ether, and 6% croton oil in diethyl ether in a ratio of 1: 4: 5: 10). After five days, croton oil-induced hemorrhoid mouse was randomly divided into seven groups and treated daily with different samples for 14 days as follows:

2.8.2. Histomorphological study of anorectal

On the 14th day, 1 h after the treatment, rat anorectal histology samples were obtained by fixing rat anorectal biopsies into 10% formalin solution, paraffin embedding, dissection and hematoxylin-eosin staining. All sample histology slides were observed using an Inverted system microscope, IX71-IX2 series optical microscope, the DP71 camera, and Cell D software (Olympus; Shinjuku-ku, Tokyo, Japan). Histological parameters such as the number of inflammatory cells, congestion, bleeding, vasodilation, and necrosis are observed in the histological preparations of anorectal tissue (Nishiki et al., 1988; Azeemuddin et al., 2014).

2.9. Statistical analysis

The results are expressed as means ± SD (standard deviation of the mean). Statistical differences between groups were estimated using a one-way analysis of variance (ANOVA) with Tukey's test and were considered statistically significant at $p < 0.05$.

3. Result

3.1. Chemical content in GPLE

The percentage of GPLE obtained with the extraction process was 17.2% (w/w). Ozaki et al. suggest that flavonoids are responsible for the anti-inflammatory activity of the extract (Ozaki et al., 1989). Therefore, the total flavonoid and total polyphenols levels in GPLE were evaluated at first. The results indicated a presence of a significant amount of total flavonoids (16.3 ± 0.79 mg/g HE) and phenolic compounds (428.3 ± 18.01 mg/g GAE) in GPLE.

3.2. Astringent properties and PRT activity of GPLE

Plasma coagulation time was determined as the ability of the extract to stop bleeding. In the present result, the PRT of GPLE and control were

Table 1
Mice grouping and treatment given to each group.

Group	Name of group	Oral			Topical		
		Vehicle	Drug	Sample	Base	Drug	Sample
		0.05% of CMC-Na	betamethasone tablets suspended in vehicle	GPLE suspended in vehicle	cream base	betamethasone cream	10% GPLE in a cream base
Group I	C group	1 ml/kg	–	–	250 mg	–	–
Group II	OB group	–	(0.065 mg/kg)	–	–	–	–
Group III	TB group	–	–	–	–	(250 mg/kg)	–
Group IV	OTB group (combination)	–	(0.065 mg/kg)	–	–	(250 mg/kg)	–
Group V	OG group	–	–	166.4 mg/kg	–	–	–
Group VI	TG group	–	–	–	–	–	150 mg/kg
Group VII	OTG group (combination)	–	–	166.4 mg/kg	–	–	150 mg/kg

(−): not given.

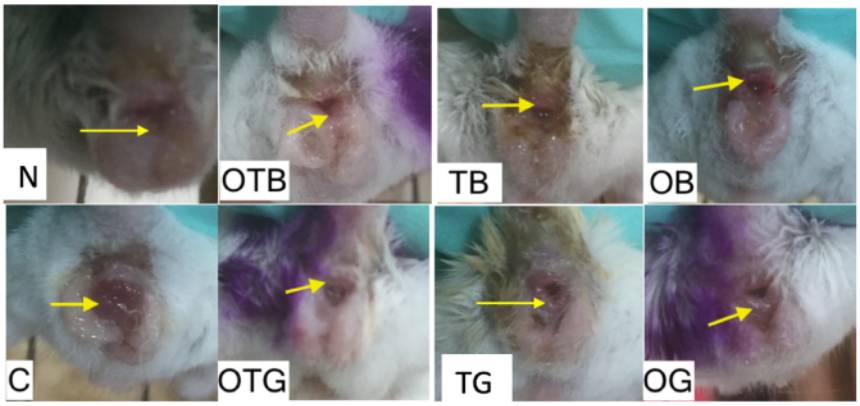

Fig. 2. Photographs of hemorrhoids of animals treated with betamethasone and GPLE (See Table 1) on days 14 after sample application. (N): normal mouse without induction of hemorrhoid.

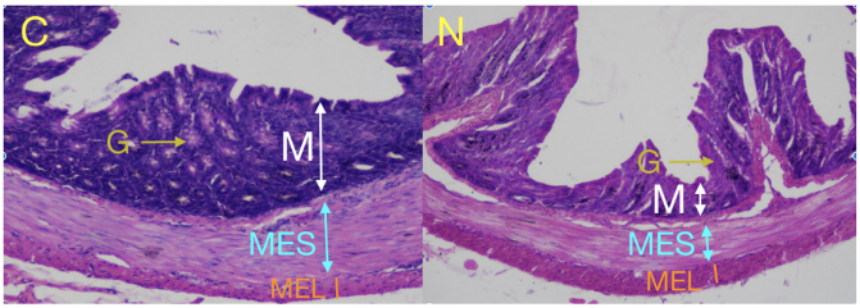

Fig. 3. Hematoxylin and eosin staining of histological sections of hemorrhoid anorectal from croton oil-induced (C) and uninduced mouse (N). Magnification 200X. M (mucosa), MEL (outer longitudinal layer of *muscularis externa*), MES (inner circular layer of *muscularis externa*), G (goblet cell).

0.46 and 2.12 min, respectively. GPLE showed a significant reduction of PRT to 21.7% (78.3% reduction) of the control (Table 2).

3.3. Antioxidant activity of GPLE

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical is a stable free radical with intense purple color and reacts with a hydrogen atom to form DPPH (1,1-diphenyl-2-picrylhydrazine, pale yellow). Trolox is an analog of vitamin E and is used as a reference compound. The dose-dependency of GPLE was evaluated by in vitro assay, and the result was shown in Fig. 1. Both Trolox and GPLE showed an almost linear response with the correlation coefficient value (r) > 0.95 (Table 3).

The IC_{50} value of GPLE was 143.0 ± 1.04 μg/mL, while Trolox was 13.8 ± 0.46 μg/mL. The activity of GPLE was weaker than Trolox. However, given that the GPLE was a crude mixture, the activity appeared was promising for the efficient control of oxidative stress

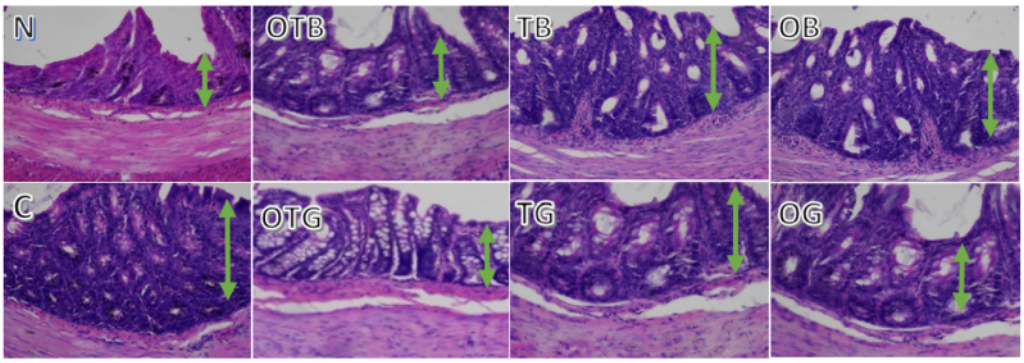

Fig. 4. Hematoxylin and eosin staining of histological sections of hemorrhoid anorectal from animals treated with C, OTB, OTG, TB, TG, OB, OG, and a normal mouse (N) on days 14 after croton oil-induction. Magnification 200X. C (control), OTB (oral and topical betamethasone), TB (topical betamethasone), OB (oral betamethasone), OTG (oral and topical GPLE), TG (topical GPLE), OG (oral GPLE).

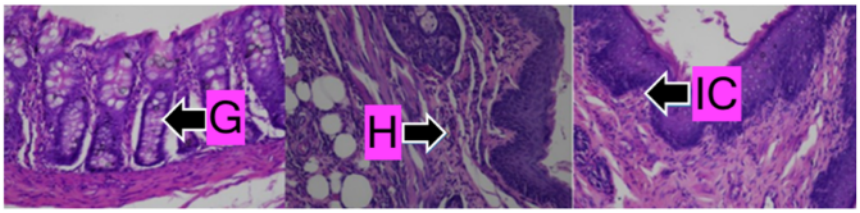

Fig. 5. Hematoxylin and eosin staining of histological sections of hemorrhoid anorectal on the mucosa portion from animals on days 14 after the croton oil-induction. Magnification 200X. G (goblet cell), H (hemorrhagic area), IC (inflammatory cells).

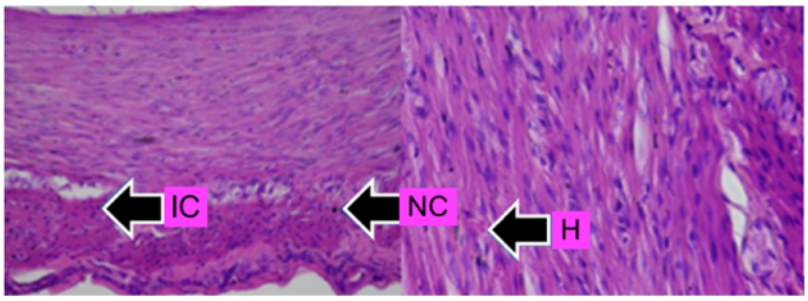

Fig. 6. Hematoxylin and eosin staining of histological sections of hemorrhoid anorectal on the *muscularis externa* portion from animals on days 14 after croton oil-induction. Magnification 200X. H (hemorrhagic area), IC (inflammatory cells), NC (necrotic cells).

during the progression of hemorrhoids.

3.4. Analysis of anorectal histopathology of in mice hemorrhoid model

The mice pathological model of hemorrhoids was created by the application of croton oil at the anorectal region for five days. The symptoms of hemorrhoids were recorded in photographs after 14 days of treatment with each prescription (Table 1 and Fig. 2). The vehicle treatment group, after the induction of hemorrhoids, showed severe inflammation as a red swelling (C). On the other hand, both groups treated with the positive control, betamethasone (OTB, TB, and OB), or GPLE (OTG, TG, and OG) showed significant improvement. The combination of oral and topical applications (OTB and OTG) seems better than single usages such as TB, OB, TG, and OG.

Histological analysis was then carried out quantitatively. The thickness of mucosa and *muscularis externa* were measured from the photograph to describe the effect of the treatments. The five days croton oil treatment apparently induced inflammation and hypertrophy of goblet cells (G), mucosa (M), and *muscularis externa* (MES) in the control group (C) compared to the normal mouse (N) (Fig. 3).

The treatment with betamethasone (B) or GPLE (G) significantly reduced the thickness of the mucosa (Fig. 4 and Table 4) and *muscularis externa* (Table 5). In Fig. 4, croton oil caused an increase in the mucosal thickness of mice anorectal into 360.7 ± 39.5 μm (C), which is 3.6 times thicker than normal mice (108.0 ± 7.1 μm) (N). After oral and topical combination application of GPLE extract (OTG) for 14 days, there was a decrease of mucosal thickness to 151.0 ± 30.8 μm, whereas single topical (TG) or oral (OG) application of extract showed a decrease to 233.7 ± 35.4 μm and 237.4 ± 26.0 μm, respectively. These ameliorations in mucosal thickness by GPLE application showed significant differences compared with the control group (C). In addition, the oral and topical combination of GPLE (151.0 ± 30.8, OTG) was comparable to the clinical drug betamethasone (135.2 ± 27.6, OTB) (Table 4).

Other symptoms were seen in the mucosa and *muscularis externa* as hemorrhage and infiltration of inflammatory cells (Figs. 5 and 6), which indicated the severity of inflammation in the mucosa and *muscularis externa* in the anorectal region. The area of hemorrhage and the number of inflammatory cells were then counted per 10,000 μm^2 for evaluating the effectiveness of GPLE application (Tables 4 and 5). All treatments showed a significant reduction in hemorrhage area and the number of inflammatory cells compared to the control group (C) (1653.0 ± 103.8 μm^2/10,000 μm^2 and 33.6 ± 3.6 cells/10,000 μm^2 in the mucosa, and 369.0 ± 34.1 μm^2/10,000 μm^2 and 7.08 ± 0.70 cells/10,000 μm^2 in *muscularis externa*, respectively) (Tables 4 and 5). The combination therapy of GPLE (OTG) dramatically decreased these symptoms both in the mucosa and *muscularis externa* (212.1 ± 14.0 μm^2/10,000 μm^2 and 13.0 ± 6.0 cells/10,000 μm^2 in the mucosa, and 67.2 ± 7.1 μm^2/10,000 μm^2 and 1,00 ± 0.14 cells/10,000 μm^2 in *muscularis externa*, respectively), even though the single applications of GPLE as topical or oral were also significantly effective (Tables 4 and 5). Besides, the combination therapy of GPLE (OTG) also reduced the number of necrotic cells significantly (0.52 ± 0.11 cells/10,000 μm^2) compared to the control (2.88 ± 0.22 cells/10,000 μm^2) (Fig. 6 and Table 5).

4. Discussion

A famous Greek physician, Hippocrates, first used the word hemorrhoids from the Greek words, *haema* (blood) and *rhoos* (flow) because of the characteristic symptom of bleeding from the anus (Leff, 1987). The prevalence of hemorrhoids is estimated from 4 to 55% of the population, with no significant difference between males and females (Yamana, 2017). Because of a kind of embarrassment, patients tend to relieve their symptoms by self-medication with over-the-counter (OTC), herbal, and ethnomedicines (Donmez 2020). The leaves of *G. pictum* are traditionally used to treat hemorrhoids in Indonesia (Heyne, 1987; Ministry of Health, 2010). However, sufficient scientific evidence has not been reported so far. Therefore, anti-hemorrhoidal effect of *G. pictum* was evaluated scientifically in this study, focusing on its antioxidant, astringent, fibrin-forming, and histomorphological amelioration in a croton oil induced hemorrhoid mouse model.

Oxidative stress by reactive oxygen species (ROS) contributes to the initiation and development of various diseases, including hemorrhoids.

Table 2
Effect of GPLE on astringent properties and plasma recalcification time.

Sample	Astringent properties	Plasma Recalcification Time (PRT) (min)
Control	no coagulation	2.12 ± 0.19*
GPLE (5%)	coagulation	0.46 ± 0.19*
Heparin	NA	no coagulation
difference (%)	NA	78.3

Values were expressed as mean ± SD (n = 5); *P < 0.05 using T-tests; NA (not available).

Table 3
Data used to calculate the IC_{50} in DPPH assay of GPLE.

Samples	Slope	Intercept	r^2	Range concentration	IC_{50} (μg/mL)
GPLE	0.2807	9.7335	0.9921	40-200 (μg/mL)	143.0 ± 1.04[b]
Trolox	2.5376	15.3030	0.9922	4-20 (μg/mL)	13.8 ± 0.46[a]

Values are expressed as mean ± SD (n = 5); means in the same column followed by different letters are significantly different at P < 0.05 using T-tests.

Table 4
Histopathology evaluation of anorectal mucosa of mice.

Sample	Thickness of mucosa (μm)	Hemorrhage area (μm^2/10,000 μm^2)	Number of inflammatory cells/10,000 μm^2
N	108.0 ± 7.1 [b]	9.3 ± 1.4 [b]	4.0 ± 0.7 [b]
C	360.7 ± 39.5 [a]	1653.0 ± 103.8 [a]	33.6 ± 3.6 [a]
OTB	135.2 ± 27.6 [b]	134.3 ± 8.2 [c]	7.4 ± 1.5 [b]
OTG	151.0 ± 30.8 [b]	212.1 ± 14.0 [d]	13.0 ± 6.0 [cd]
TB	216.4 ± 70.5 [c]	466.1 ± 9.7 [f]	12.2 ± 2.4 [c]
TG	233.7 ± 35.4 [c]	599.2 ± 65.9 [g]	20.6 ± 5.2 [e]
OB	237.4 ± 26.0 [c]	399.2 ± 66.7 [e]	16.4 ± 2.1 [de]
OG	267.7 ± 37.5 [c]	850.1 ± 13.7 [h]	26.6 ± 3.1 [f]

Values are expressed as mean ± SD (n = 5); means in the same column followed by different letters are significantly different at P < 0.05 using Tukey's multiple range tests.

Thus a sufficient amount of antioxidants is essential to prevent damage to anorectal tissue (Saad and Lamia, 2009; Faujdar et al., 2018). The ethanol extract of *G. pictum* leaves (GPLE) contained significant amounts of flavonoids (16.3 ± 0.79 mg/g HE) and phenolic compounds (428.3 ± 18.01 mg/g GAE) as potential natural antioxidants. In actually, DPPH analysis confirmed the antioxidant activity of GPLE (IC_{50} = 143.0 ± 1.04 μg/mL). These results indicate that GPLE contains chemical components that are favorable for reducing oxidative stress in hemorrhoidal tissues.

As for bleeding control, it is thought that astringent activity is related to hemostatic property in the aspects of vasoconstriction and blood coagulation (Nabavizadeh et al., 2016; Ebrahimi et al., 2020; Dandjesso et al., 2012; Odukoya et al., 2009). The results of the astringency assay clearly showed the positive effect of GPLE (Table 2). In addition, plasma recalcification time (PRT), which is an important parameter of blood coagulation (Abascal and Yarnell, 2005; Ohkura et al., 2015; Ream Nayal and M Yasser Abajy, 2015), has significantly reduced by the treatment with GPLE (0.46 ± 0.19 min) compared to that of control (2.12 ± 0.19 min) (Table 2). These results suggest that GPLE has beneficial features in stopping the bleeding of hemorrhoids by astringency and coagulation activities. However, the most important thing is whether or not it works in animal models, as discussed below.

Croton oil has a strong irritant property in the skin and mucosa and is generally used to induce mice hemorrhoids. In this study, we used a croton oil-induced mouse hemorrhoid model to examine in vivo activity, focusing on the following histological parameters in the anal region, such as a thickness of the mucosa and external muscle, the number of inflammatory cells, the area of bleeding, and necrotic cell number

Table 5
Histopathology evaluation of anorectal *muscularis externa* portion of mice.

Sample	Thickness of *muscularis externa* (μm)	Hemorrhage area (μm^2/10,000 μm^2)	Number of inflammatory cells/10,000 μm^2	Number of necrosis cells/10,000 μm^2
N	121.8 ± 30.1 [b]	10.9 ± 3.8 [b]	0.08 ± 0.11 [b]	0.04 ± 0.09 [b]
C	399.9 ± 50.3 [a]	369.0 ± 34.1 [a]	7.08 ± 0.70 [a]	2.88 ± 0.22 [a]
OTB	139.8 ± 38.8 [b]	41.9 ± 8.9 [c]	0.52 ± 0.11 [c]	0.24 ± 0.09 [b]
OTG	202.1 ± 24.0 [c]	67.2 ± 7.1 [c]	1.00 ± 0.14 [c]	0.52 ± 0.11 [c]
TB	218.4 ± 53.1 [c]	183.9 ± 21.8 [d]	1.60 ± 0.37 [d]	1.16 ± 0.21 [d]
TG	219.6 ± 60.4 [c]	252.4 ± 24.9 [e]	1.96 ± 0.33 [d,e]	1.12 ± 0.11 [d]
OB	294.3 ± 30.1 [d]	229.1 ± 25.0 [e]	2.20 ± 0.80 [e]	1.28 ± 0.30 [d]
OG	328.5 ± 32.7 [d]	281.8 ± 12.0 [f]	2.08 ± 0.50 [f]	1.72 ± 0.33 [e]

Values are expressed as mean ± SD (n = 5); means in the same column followed by different letters are significantly different at P < 0.05 using Tukey's multiple range tests.

(Figs. 2–4, Tables 4 and 5). The combination of topical and oral GPLE application significantly reduced these symptoms comparable to that of the positive control, betamethasone (Figs. 2–4, Tables 4 and 5). These results strongly support the ethnomedicinal use of *G. pictum* as a treatment for hemorrhoids.

The release of inflammatory mediators such as prostaglandins, leukotrienes, TNF-α, nitric oxide, and bradykinin are induced by croton oil treatment. These factors regulate the activation of fibroblasts, endothelial cells, monocytes, lymphocytes, and neutrophils, which leads to severe inflammation and hemorrhoids (Azeemuddin et al., 2014; Faujdar et al., 2018). Some flavonoids have been reported to have anti-inmflammatory activityies (Hosek and Smejkal, 2015; Kim et al., 2004). GPLE contains various compounds including flavonoids and polyphenol, which may directly or synergistically regulate the expression and function of these inflammatory mediators, but further investigation of chemical constituents and expression analysis of mRNA and proteins is needed to unveil the detailed mechanisms of this ethnomedicine.

5. Conclusion

The ethanol extract of *Graptophyllum pictum* leaves was suggested to have a therapeutic effect on hemorrhoids by its antioxidant, anti-inflammatory and hemostatic properties. The present study validates the ethnomedicinal use of this plant against hemorrhoids and suggests its therapeutic potential as a promising anti-hemorrhoid agent.

Declaration of competing interest

There is no conflict of interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2021.114765.

Author contributions

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Credit author statement

Conceptualization: Idha Kusumawati, Methodology:Idha Kusumawati, Rohmania, Aisyah Farah Rizka, Eka Pramyrtha Hestianah, Katsuyoshi Matsunami, Investigation: Idha Kusumawati, Subhan Rullyansyah, Resources: Idha Kusumawati, Rohmania, Aisyah Farah Rizka, Eka Pramyrtha Hestianah, Data curation: Subhan Rullyansyah, Rohmania, Aisyah Farah Rizka, Eka Pramyrtha Hestianah, Writing – original draft: Idha Kusumawati, Subhan Rullyansyah, Writing – review & editing: Idha Kusumawati, Subhan Rullyansyah, Katsuyoshi Matsunami, Supervision: Idha Kusumawati, Katsuyoshi Matsunami

References

Abascal, K., Yarnell, E., 2005. Botanical treatments for hemorrhoids. Alternative Compl. Ther. 11, 285–289. https://doi.org/10.1089/act.2005.11.285.

Azeemuddin, M., Viswanatha, G.L., Rafiq, M., Thippeswamy, A.H., Baig, M.R., Kavya, K. J., Patki, P.S., Shyam, R., 2014. An improved experimental model of hemorrhoids in rats : evaluation of antihemorrhoidal activity of an herbal formulation. ISRN Pharmacol 2014, 1–7.

Christ, B., Muller, K., 1960. Zur serienmaSigen Bestimmung des Gehaltes an Flavonol-Derivaten in Drogen. Arch. Pharm. (Weinheim). 293, 1033–1042.

Čuk, Vladimir, Ščepanović, M., Krdzić, I., Kenić, M., Kovačević, B., Čuk, Vladica, 2015. Where are we now in the treatment of hemorrhoids. Acta Medica Median 54, 97–106. https://doi.org/10.5633/amm.2015.0116.

Dandjesso, C., Klotoé, J.R., Dougnon, T.V., Sègbo, J., Atègbo, J.M., Gbaguidi, F., Fah, L., Fanou, B., Loko, F., Dramane, K., 2012. Phytochemistry and hemostatic properties of some medicinal plants sold as anti-hemorrhagic in Cotonou Markets (Benin). Indian J. Sci. Technol. 5, 3105–3109.

Donmez, C., Yalcin, F.N., Boyacioglu, O., Korkusuz, P., Akkol, E.K., Nemutlu, E., Balaban, Y.H., Caliskan, U.K., 2020. From nutrition to medicine: assessing hemorrhoid healing activity of *Solanum melongena* L. via in vivo experimental models and its major chemicals. J. Ethnopharmacol. 261, 113143. https://doi.org/10.1016/j.jep.2020.113143.

Mackay, Douglas, 2001. Hemorrhoids and varicose veins: a review of treatment options. Alternative Med. Rev. 6, 126–140.

Ebrahimi, F., Torbati, M., Mahmoudi, J., Valizadeh, H., 2020. Medicinal plants as potential hemostatic agents. J. Pharm. Pharmaceut. Sci. 23, 11–23. https://doi.org/10.18433/jpps30446.

Elahi, M.F., Guan, G., Wang, L., 2014. Hemocompatibility of surface modified silk fibroin materials: a review. Rev. Adv. Mater. Sci. 38, 148–159.

Faujdar, S., Sati, B., Sharma, S., Pathak, A.K., Paliwal, S.K., 2018. Phytochemical evaluation and anti-hemorrhoidal activity of bark of Acacia ferruginea DC. J. Tradit. Complement. Med. 1–5. https://doi.org/10.1016/j.jtcme.2018.02.003.

Heyne, K., 1987. Tumbuhan Berguna Indonesia, ume 3. Yayasan Sarana Wana Jaya, Jakarta.

Hosek, J., Smejkal, K., 2015. Flavonoids as anti-inflammatory agents. In: Parnham, M. (Ed.), Encyclopedia of Inflammatory Diseases. Springer, Basel, pp. 1–17. https://doi.org/10.1007/978-3-0348-0620-6.

Jafari, S., Moradi, A., Salaritaba, A., Hadjiakhoo, A., Khanavi, M., 2010. Determination of total phenolic and flavonoid contents of Leonurus cardiaca L. In compare with antioxidant activity. Res. J. Biol. Sci. 5, 484–487. https://doi.org/10.3923/rjbsci.2010.484.487.

Jiangseubchatveera, N., Liawruangrath, B., Liawruangrath, S., Teerawutgulrag, A., Santiarworn, D., Korth, J., Pyne, S.G., 2015. The chemical constituents and the cytotoxicity, antioxidant and antibacterial activities of the essential oil of *Graptophyllum pictum* (L.) Griff. J. Essent. Oil Bear. Plants 18, 11–17. https://doi.org/10.1080/0972060X.2014.935036.

Kim, H.P., Son, K.H., Chang, H.W., Kang, S.S., 2004. Anti-inflammatory plant flavonoids and cellular action mechanisms. J. Pharmacol. Sci. 96, 229–245. https://doi.org/10.1254/jphs.CRJ04003X.

Klotoé, J.R., Dougnon, T.V., Sacramento, T.I., Dandjesso, C., Edorh, A.P., Koudokpon, H., Fanou, V.B.A., Fah, L., Atègbo, J.M., Loko, F., Dramane, K., 2012. Hemostatic potential of the sap of Musa sapientum L. (Musaceae). J. Appl. Pharmaceut. Sci. 2, 65–69. https://doi.org/10.7324/JAPS.2012.2707.

Kusumawati, I., Indrayanto, G., 2013. Natural antioxidants in cosmetics. In: Atta-ur-Rahman, F. (Ed.), Studies in Natural Products Chemistry. Elsevier B.V., Amsterdam, pp. 485–505. https://doi.org/10.1016/B978-0-444-59603-1.00015-1.

Kusumawati, I., Maat, S., Hafid, A.F., 1997. The influence of etanolic extract of Graptophyllum pictum (L. Griff Leaves on Non Spesific Immune Responces.

Kusumawati, I., Dyatmiko, W., Santosa, M.H., Maat, S., 2002. The effect of ethanol extract of the leaf of Graptophyllum pictum (L.) Griff againts phagositosis function and formation of immunoglobulin. Airlangga J. Pharm. 2, 76–79.

Kusumawati, I., Kurniawan, K.O., Rullyansyah, S., Prijo, T.A., Widyowati, R., Ekowati, J., Hestianah, E.P., Maat, S., Matsunami, K., 2018. Anti-aging properties of Curcuma heyneana Valeton & Zipj: a scientific approach to its use in Javanese tradition. J. Ethnopharmacol. 225 https://doi.org/10.1016/j.jep.2018.06.038.

Leff, E., 1987. Hemorrhoids: current approaches to an ancient problem. Postgrad. Med. 82 (7), 95–101. https://doi.org/10.1080/00325481.1987.11700060.

Levang, P., Foresta, H. de, 1991. Economic Plants of Indonesia : a Latin, Indonesian, French and English Dictionary of 728 Species, Orstom and SEAMEO BIOTROP. ORSTOM and SEAMEO BIOTROP.

Loder, P.B., Kamm, M.A., Nicholls, R.J., Phillips, R.K.S., 1994. Haemorrhoids: pathology, pathophysiology and aetiology. Br. J. Surg. 81, 946–954. https://doi.org/10.1002/bjs.1800810707.

Lohsiriwat, V., 2012. Hemorrhoids: from basic pathophysiology to clinical management. World J. Gastroenterol. 18 https://doi.org/10.3748/wjg.v18.i17.2009, 2009–2017.

Ministry of Health, R.I., 2010. Guidelines for the Use of Herbal Medicines in Family Health Care, vol. 115.

Misra, M.C., Imlitemsu, 2005. Drug treatment of haemorrhoids. Drugs 65, 1481–1491. https://doi.org/10.2165/00003495-200565110-00003.

Mounsey, A.L., Halladay, J., Sadiq, T.S., 2011. Hemorrhoids. Am. Fam. Physician 84, 204–210.

Nabavizadeh, M.R., Zargaran, A., Moazami, F., Askari, F., Sahebi, S., Farhadpoor, A., Faridi, P., 2016. Comparison of the hemostatic activity of Quercus persica Jaub . & Spach . (oak) with ferric sulfate in Bony crypts. J. Evid. Based. Complementary Altern. Med. 21, 34–38. https://doi.org/10.1177/2156587215593378.

Nan, M., Pintea, A., Bunea, A., Eşianu, S., Tămaş, M., 2012. HPLC analysis of carotenoids from inula helenium L. flowers and leaves. FARMACIA 60, 501–509.

Nayal, Ream, M Yasser Abajy, S.T., 2015. In vitro investigating of the hemostatic effect of some medicinal plants. Res. J. Aleppo Univ. 100, 7–20.

Nishiki, K., Nishinaga, K., Kudoh, D., Iwa, K., 1988. [Croton oil-induced hemorrhoid model in rat : comparison of anti- inflammatory activity of diflucortolone valerate with other glucocorticoids]. Nihon Yakurigaku Zasshi 92, 215–225.

Odeh, I., Abbadi, J., Obeyat, L., Qabbajeh, M., Hroub, A., 2014. Effect of harvesting date and variety of date palm on antioxidant capacity , phenolic and flavonoid content of date palm (Phoenix Dactylifera). J. Food Nutr. Res. 2, 499–505. https://doi.org/10.12691/jfnr-2-8-11.

Odukoya, O., Sofidiya, M., Ilori, O., Gbededo, M., 2009. Hemorrhoid therapy with medicinal plants: astringency and inhibition of Lipid peroxidation as key factors. Int. J. Biol. Chem. 3, 111–118.

Ohkura, N., Yokouchi, H., Mimura, M., Nakamura, R., Atsumi, G., 2015. Screening for hemostatic activities of popular Chinese medicinal herbs in vitro. J. Intercult. Ethnopharmacol. 4, 19. https://doi.org/10.5455/jice.20141128032845.

Ozaki, Y., Sekita, S., Soedigdo, S., Harada, M., 1989. Anti-inflammatory effect of Graptophyllum pictum (L.). Griff. Chem. Pharm. Bull. 37, 2799–2802. https://doi.org/10.1248/cpb.37.3229.

Ramdhan, B., Chikmawati, T., Waluyo, E.B., 2015. Ethnomedical herb from cikondang indigenous village, district Bandung. J. Biodivers. Environ. Sci. J. Bio. Env. Sci 6, 277–288.

Saad, K.A.-F., Lamia, A.M.A.-M., 2009. A new keynote of β – thalassemia major patients as oxidative stress accompanying. Med. J. Babylon 6, 659–666.

Sardiñas, C., Arreaza, D.D., Osorio, H., 2016. Changes in the proportions of types I and III collagen in hemorhoids : the sliding anal lining theory. J. Coloproctology 36, 124–129. https://doi.org/10.1016/j.jcol.2016.04.003.

Singleton, V.L., Rossi, J.A.J., 1965. Colorimetry to total phenolics with phosphomolybdic acid reagents. Am. J. Enol. Vinic. 16, 144–158.

Sun, Z., Migaly, J., 2016. Review of hemorrhoid disease: presentation and management. Clin. Colon Rectal Surg. 29, 22–29. https://doi.org/10.1055/s-0035-1568144.

Yamana, T., 2017. Japanese practice guidelines for anal disorders II. Anal fistula. J. Anus, Rectum Colon 1, 89–99. https://doi.org/10.23922/jarc.2018-009.

Yildirim, B.A., Kordali, S., Yildirim, S., Yildirim, F., 2017. Protective effect of polygonum cognatum meissn ethanolic extract on experimental hemorrhoid in rats. Int. J. Curr. Res. 9, 46213–46218.

Zaman, M.M., Dowla, R., Rahman, S.S., Islam, T.M.N., Tareq-Al-Hossain Rahman, A., Chowdhury, F.R., Sharmin, S., 2015. Hemorrhoids. Info Med. 12, 3–7.

Glossary

$AlCl_3$: aluminium chloride
ANOVA: analysis of variance
C: control
$CaCl_2$: calcium chloride
CMC: carboxymethylcellulose
DMSO: dimethyl sulfoxide
DPPH: 1,1-diphenyl-2-picrylhydrazyl
GAE: gallic acid equivalent
GPL: Graptophyllum pictum leaves
GPLE: Graptophyllum pictum leaves extract
H: hemorrhagic area
HE: hyperoside equivalent
IC: inflammatory cells
IC50: 50% inhibitory concentration
ICR: Institute of Cancer Research
N: normal
NC: necrotic cells
OB: oral betamethasone
OG: oral *Graptophyllum pictum* leaves extract
OTB: oral-topical betamethasone
OTG: oral-topical *Graptophyllum pictum* leaves extract

PPP: Plasma Poor platelet
PRT: Plasma recalcification time
ROS: Reactive oxygen species
SEM: standard error of the mean
TB: topical betamethasone
TG: topical *Graptophyllum pictum* leaves extract

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