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Gratophyllum pictum (L.) Griff Extract as Anti-Inflammatory on Wistar Rats With Experimental Hemorrhoids

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Background: The ethanol *Graptophyllum pictum* (L.) Griff extract (EGPE) exerts an anti-inflammatory effect on inflammatory-induced rat feet and has been used as a traditional medicine in Indonesia for treating hemorrhoids.

Aim: The aim of this study was to evaluate the effects of EGPE as an anti-inflammatory on Wistar rats with experimentally induced hemorrhoids.

Methods: Twenty-eight Wistars were allocated into 4 groups. Groups 2, 3, and 4 hemorrhoids were induced with 6% croton oil into the anus, whereas group 1 was not induced. Groups 1 and 2 were given physiologic saline, and groups 3 and 4 were given EGPE 100 and 300 mg/kg body weight (BW), respectively. On day 9, blood was aspirated from the retro-ocular region for the examination of serum interleukin (IL)-6, cyclooxygenase-2 (COX-2), and tumor necrosis factor (TNF)-a (ELISA method) and serum glutamic oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), urea, and creatinine levels. The anus was prepared for microscopic examination to count leucocytes.

Results: The induction of 6% croton oil significantly increased TNF-α, IL-6, COX-2, and leucocyte count. An EGPE dose of 100 mg/kg BW significantly decreases TNF-α, IL-6, COX-2, and leucocyte counts, whereas a dose of 300 mg/kg BW significantly decreased TNF-α and leucocyte count. SGOT, SGPT, blood urea, and creatinine levels were not significantly different among groups.

Conclusion: The EGPE dose of 100 mg/kg BW has anti-inflammatory effects on hemorrhoids by suppressing IL-6, COX-2, TNF-α, and total leucocytes, whereas the

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inflammatory effects of 300 mg/kg BW reduced TNF-α and total leucocytes. EGPE is safe for the kidneys and liver.

Key words: Graptophyllum pictum (L.) Griff – IL-6 – TNF-α – COX-2 – Hemorrhoids

The first-line treatment of hemorrhoids is based on its grade.[1,2] The modality of treatment of grades 1 and 2 and small grade 3 hemorrhoids consists of dietary modification, with high fiber and plenty of water intake, lifestyle modifications, and medical treatments (phlebotonic and anti-inflammatory drugs). There are various types of medical treatments for hemorrhoids, but micronized purified flavanoid fraction (MPFF) that consists of 90% diosmin and 10% hesperidin given orally has already been used extensively. Randomized controlled trials (RCTs)[3,4] and meta-analysis of several RCTs[5] have reported that MPFF reduces swelling, discharge, pain, and bleeding significantly in medically treated hemorrhoids and also reduces postoperative bleeding and pain in comparison with the group without MPFF.[6] MPFF already exists in the Indonesian market, as an imported regiment, but it is not included in the national formularies, and it is not allowed to be given to a patient who is covered by national insurance.

Justicia picta, Purple leaf, or *Graptophyllum pictum* (L.) Griff has already been used as a form of traditional medicine to treat hemorrhoids in Indonesia,[7] but a study regarding the mechanism of action and its safety has never been reported. Ethanol *Graptophyllum pictum* extract (EGPE) contains alkaloid, glycoside, pectin, formic acid, steroid, saponin, tannin, flavonoid, and alcohol.[7] Flavanoids from EGPE are proven to have an anti-inflammatory effect on inflammation-induced rat feet comparable to indomethacin.[8] In this article, the effects of EGPE as an anti-inflammatory for experimental Wistar rat hemorrhoids will be reported.

Materials and Methods

This was an experimental study using Wistar rats, which received induced hemorrhoids using croton oil. The anti-inflammatory effects of EGPE on experimental hemorrhoids were evaluated using the parameters of serum interleukin (IL)-6, COX-2, tumor necrosis factor (TNF)-α, and total leucocyte count from the anus of Wistar rats. The secondary outcomes of this study were to investigate the toxicity of EGPE to the liver and kidneys. This study was approved by the Ethical Committee Board of Faculty of Medicine, Diponegoro Medical Faculty, Dr Kariadi Hospital, Semarang, Central Java, Indonesia.

EGPE

Graptophyllum pictum (GP) is a shrub plant and a member of the Acanthaceae family, which is believed to be native to New Guinea,[9] but now can be found in tropical countries including Indonesia. GP leaves were provided from the Sido Muncul herbal medicine factory farm, in Semarang, Indonesia, and the extraction processes were also done in this factory. GP powder was extracted with 70% ethanol using a soxhlet extractor, which was then concentrated in a vacuum container to achieve 95% concentration, and stored at 15°C to 20°C.[10] The doses of EGPE were 100 and 300 mg/kg body weight (BW), given twice daily.[8,11]

Croton oil

The croton oil was provided online from Sigma Aldrich Company (catalog no. C6719-10G; St Louis, Missouri). The Croton oil for anal induction was prepared by mixing deionized water, pyridine, diethyl ether, and 6% croton oil in diethyl ether at a ratio of 1:4:5:10. After overnight fasts, sterile cotton swabs (4 mm in diameter) were immersed in 100-μL croton oil preparations and then were inserted into the anus 15 mm from the anal opening in all the Wistar mice and kept in the anus for 30 seconds, once a day for 3 consecutive days.[12]

Animals

The animals were healthy male adult Wistar rats, age of 10 to 12 weeks, with a weight of approximately 200 g. The animal was excluded if during the study it appeared to be sick or dying. The Wistar rats were obtained from the animal house unit of the Lembaga Pengembangan Penelitian Terapan (LPPT) University of Gajahmada, Yogjakarta. The studies were also done at LPPT, an accredited research laboratory (ISO 17025:2005). The Wistar rats were maintained in wire bottom cages at 22 ± 3°C and

50% to 60% humidity under a 12-hour–12-hour light-dark cycle for at least 1 week before the experiment. The cages were maintained according to standard housing conditions, and access to standard diet and water was provided ad libitum during the experiment. All of the animal care criteria prepared by the National Academy of Sciences and outlined in the Guide for the Care and Use of Laboratory Animals were applied throughout the experiment.[13] The experimental protocol was approved by the Ethical Committee of Faculty of Medicine, University of Diponegoro, dr Kariadi Hospital Semarang Indonesia.

Experimental design

Rats that met the inclusion criteria were randomly divided into 4 different groups. Group 1 did not have induction of hemorrhoids by croton oil and were given physiologic saline orally (negative control). Group 2, 3, and 4 had hemorrhoids induced with croton oil, and on day 4 were given physiologic saline (positive control), EGPE 100 mg/kg BW, and EGPE 300 mg/kg BW, respectively, for 5 consecutive days. Termination and evaluation of the variables were executed on day 9. Blood samples were taken from the retro-orbital sinus, and then under ether anesthesia, neck dislocation was performed. The anus, containing internal and external sphincter, was then resected, with the margin 2 cm wide from the outer border of the anus.[14] The specimens were prepared for microscopic examination. Specimens were as thick as 6 μm and were stained with hematoxylin and eosin to evaluate leucocyte count. The blood was prepared for the examination of serum TNF-α, IL-6, and COX-2 using the ELISA method and the blood levels of SGOT, SGPT, urea, and creatinine. Animal manipulation and ELISA examination were completely done in LPPT. Leucocyte count on 400 magnifications of anal specimens was done at the Anatomical Pathology of National Diponegoro Hospital, Faculty of Medicine, Diponegoro University.

Statistical analysis

TNF-α, SGOT, SGPT, blood urea, and creatinine levels were normally distributed. Analysis of variance (ANOVA) and post hoc least significant difference were used for statistical analysis. The Wistar weight, serum IL-6, COX-2, and leucocyte count were non-normally distributed; therefore, nonparametric Kruskal–Wallis (KW), and Mann–Whitney (MW) tests were used to testing the differences.

Results

All Wistar rats were still in good health until the end of the study and were included in the statistical analysis. The mean (±SD) of the Wistar weight (in grams) of group 1, 2, 3, and 4 was 219.61 (26.57), 173.84 (13.37), 177.62 (14.59), and 171.70 (13.10), respectively (KW, $P = 0.009$). On the MW test, the significant difference was only for group 1 compared with other groups.

The mean (±SD) serum TNF-α concentration (pg/mL) of groups 1, 2, 3, and 4 was 147 (42), 410 (147), 262 (26), and 293 (106), respectively (ANOVA, $P = 0.000$). On post hoc least significan difference, it was significantly higher in group 2 in comparison with group 1 ($P = 0.000$) and significantly lower in groups 3 and 4 compared with group 2 ($P = 0.007$ and 0.028, respectively). In group 4, the level of TNF-α was lower compared with group 3 but was not statistically significant ($P = 0.548$; Fig. 1A).

The mean (±SD) of serum IL-6 concentration (pg/mL) of groups 1, 2, 3, and 4 was 220 (10), 433 (106), 199 (54), and 306 (118), respectively (KW test, $P = 0.005$). On the MW test, induction of 6% croton oil in Wistar rats significantly stimulated the production of IL-6 (group 1 versus group 2, $P = 0.002$). Treatment with EGPE 100 mg/kg BW reduced the IL-6 serum significantly (group 3 versus. group 2, $P = 0.003$), but it was not significant for EGPE 300 mg/kg BW (group 4 versus group 2, $P = 0.277$; Fig. 1B).

The mean (±SD) of serum COX-2 concentration (pg/mL) of groups 1, 2, 3, and 4 was 405 (34), 595 (68), 266 (158), and 441 (197), respectively (KW test, $P = 0.005$). On the MW test, induction of 6% croton oil in the anus of Wistar rats significantly stimulated the production of serum COX-2 (group 1 versus group 2, $P = 0.002$). Treatment of EGPE 100 mg/kg BW reduced serum COX-2 significantly (group 2 versus group 3, $P = 0.004$), but it was not significant for EGPE 300 mg/kg BW (group 2 versus group 4, $P = 0.085$; Fig. 2A).

The mean (±SD) of leucocyte count in the anal specimens of group 1, 2, 3, and 4 was 1711 (39), 2017 (200), 1800 (96), and 1642 (61) respectively, (KW test, $P = 0.003$). The total leucocyte count in the anal specimens was significantly higher after 6% croton oil induction in the anus of Wistar rats in comparison with no induction (MW test of group 1 versus group 2, $P = 0.018$). The total leucocyte count in the

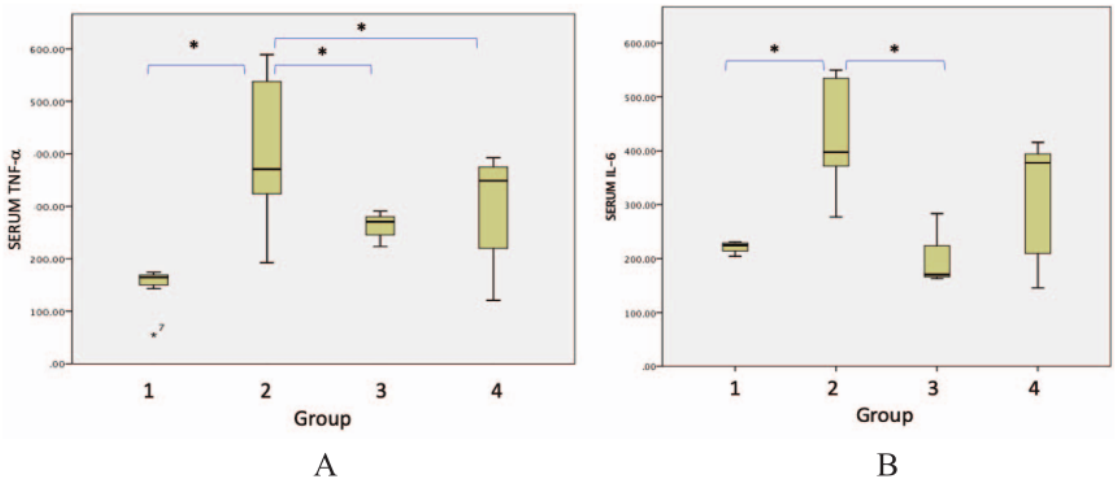

Fig. 1 Boxplot of serum (A) TNF-α and (B) IL-6 of group 1, 2, 3, and 4. *Statistically significant.

anal specimens was significantly lower in EGPE at both 100 and 300 mg/kg BW compared with the positive control group (groups 3 and 4 versus 2, $P = 0.040$ and $P = 0.004$, respectively). The dose of 300 mg/kg BW was better at suppressing total leucocyte count than the 100-mg/kg BW dose (group 3 versus 4, $P = 0.007$; Fig. 2B.

The mean (±SD) creatinine level (mg/dL) of group 1, 2, 3, and 4 was 0.27 (0.06), 0.29 (0.04), 0.27(0.06), and 0.26 (0.03), respectively (ANOVA, $P = 0.809$) . The mean (±SD) urea level (mg/dL) of groups 1, 2, 3, and 4 was 34.30 (2.82), 35.11(3.29), 33.44 (5.07), and 30.71 (4.08), respectively (ANOVA, $P = 0.200$). The mean (±SD) serum SGOT level (U/L) of groups 1, 2, 3, and 4 was 109.90 (21.48), 138.61(37.20), 130.71 (76.27), and 91.48 (25.44), respectively (ANOVA, $P = 0.234$). The mean (±SD) serum SGPT level (U/L) of groups 1, 2, 3, and 4 was 61.01 (8.79), 53.64 (11.73), 51.24 (13.04), and 47.10 (9.94), respectively (ANOVA, $P = 0.145$; Fig. 3.

Discussion

The 4 groups of the Wistar rats were comparable regarding sex, food and water intake, cage, and environment. A group without induction was

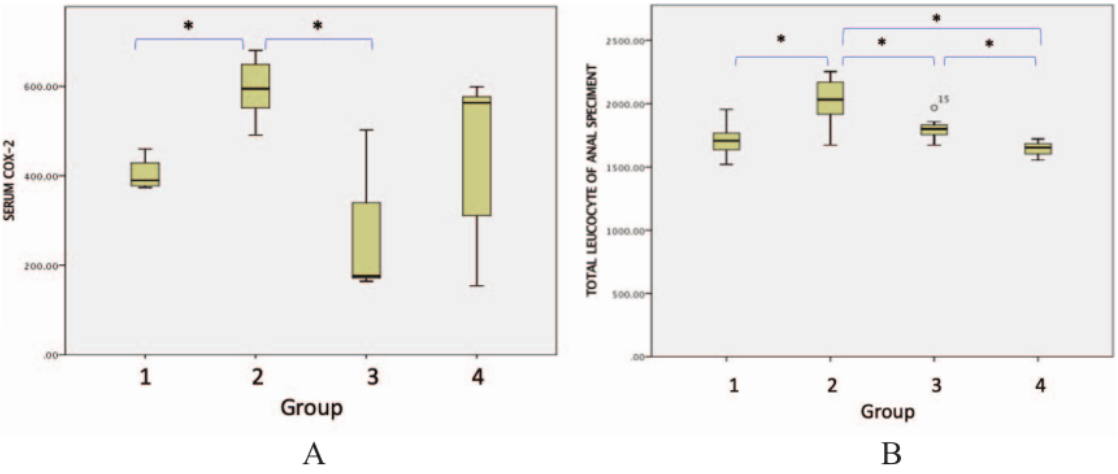

Fig. 2 Boxplot of (A) serum COX-2 and (B) leucocyte count in the anal specimens. *Statistically significant.

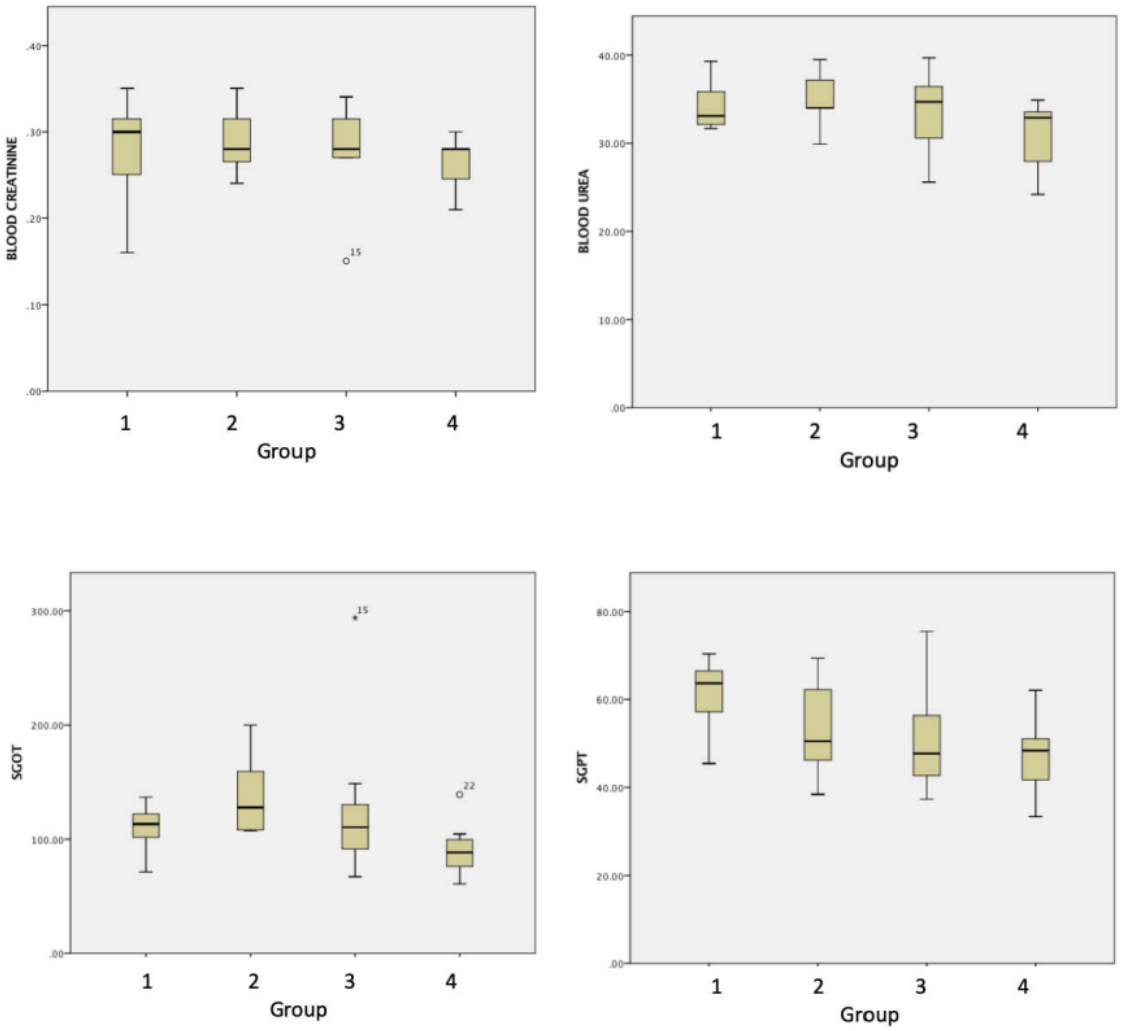

Fig. 3 Boxplot of blood creatinine, urea, SGOT, and SGPT levels of groups 1, 2, 3, and 4.

significantly heavier in comparison with other groups; meanwhile, the was no difference among groups 2, 3, and 4. This did not cause a serious problem because the intention of using group 1 was to learn the effects of croton oil induction on the inflammation reaction compared with group 2. The anti-inflammatory effects of EGPE 100 mg/kg BW (group 3) and 300 mg/kg BW (group 4) were compared with group 2.

Irritation by croton oil may damage the mucous cell. The necrotic cell will release alarmin that can induce innate immunity by activating inflammation-related pathways.[15] Alarmin through the Toll-like receptor entering the immunologic cells may stimulate nuclear factor-κB to produce proinflammatory cytokine-like TNF-α, IL-1, IL-12, and type 1 interferon (IFN).[16] These cytokines initiate a cascade of other inflammatory cytokines and chemokines such as IL-6, IL-8, and IFN-γ.[17] In this study, induction of 6% croton oil significantly increases serum TNF-α, IL-6, COX-2, and total leucocyte count in the anal specimens, indicating that contact of anal mucosae with 6% croton oil induced the development of inflammation. This is in accordance with previous research.[12,14,18]

TNF-α is a proinflammatory cytokine that, together with IL-1, will further stimulate the production of adhesion molecules that cause leucocytes to be trapped in blood vessels and then move to the inflammatory tissue. These leucocytes will be the main effector of inflammation whose goal is to eliminate bacteria, foreign bodies, or dead tissue.[19] Serum TNF-α will decrease in the course of healing.[20] In this study, serum TNF-α levels were significantly decreased in EGPE 100 and 300 mg/kg BW, which means that EGPE promotes the healing of inflammation through decreasing serum TNF-α.

IL-6 is produced in response to infection or tissue injury. After production of IL-6, it will immediately go from the bloodstream to the liver and induce the production of active proteins such as C-reactive protein. During the healing of acute infection, IL-6 will decrease.[21] In this study, EGPE 100 mg/kg BW decreased serum IL-6 levels significantly compared with positive control groups. This result showed that EGPE 100 mg/kg BW promotes healing from inflammation by decreasing IL-6.

COX-2 has an inflammatory influence and has a dominant role in the formation of prostaglandins in inflammation.[22] COX-2 expression was significantly higher in hemorrhoid tissue than in normal mucosal and submucosa rectum tissues.[23] In this study, EGPE 100 mg/kg BW significantly decreased the level of COX-2. This means that EGPE 100 mg/kg BW promotes healing of inflammation through a decrease in COX-2.

In acute inflammation, the number of leucocytes will increase, and in the repair process, the number will decrease.[24] In this study, the leucocyte counts were significantly decreased in both EGPE doses (100 and 300 mg/kg BW) compared with the control group. The dose of 300 mg/kg BW was better at suppressing total leucocytes than the 100-mg/kg BW dose. This result shows that EGPE promotes healing from inflammation through decreasing the leucocyte count in the anal tissue and the 300-mg/kg BW dose is the best. This result is not in line with data that decreasing the inflammatory markers, IL-6, COX-2, and TNF-α, was much better at the 100 compared with the 300-mg/kg BW dose. Possible explanations are that the inflammatory markers were not produced only by leucocytes. Several cells, such as the endothelium, fibroblasts, mesenchymal cells, and many other cells also produce proinflammatory markers.[19,21]

There were no significantly differences regarding SGOT, SGPT, blood urea, and creatinine levels among the groups. This result shows that EGPE is safe for the liver and kidneys. Another study showed that EGPE has a nephron-protective activity against nephrotoxic effects caused by gentamycin.[10]

In conclusion, EGPE at a dose of 100 mg/kg BW has anti-inflammatory effects in experimental hemorrhoids in Wistar rats, which can suppress serum IL-6, COX-2, and TNF-α, and leucocytes in the anal canal tissue; meanwhile, a dose of 300 mg/kg BW has an anti-inflammatory effect only through decreasing TNF-α and total leucocytes. EGPE is also safe for the kidneys and liver. Because MPFF nowadays is used largely as an anti-hemorrhoid medicine, further studies comparing EGPE and MPFF should be done. A clinical study should be done to confirm whether EGPE is also effective as an anti-inflammatory and safe for human hemorrhoids.

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