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Original Article

Role of micronize purified flavonoid fraction and ethanol *Graptophyllum pictum* extract on experimental anal ulcer healing. Study on Wistar rat

Sigit Adi Prasetyo[a], *Yan Wisnu*[a], *Eriawan Agung Nugroho*[a], *Edi Dharmana*[b], *Neni Susilaningsih*[c], *Ignatius Riwanto*[a,*]

[a] *Diponegoro University, Faculty of Medicine, Department of Surgery, Semarang, Indonesia*
[b] *Diponegoro University, Faculty of Medicine, Department of Parasitology and Immunology, Semarang, Indonesia*
[c] *Diponegoro University, Faculty of Medicine, Department of Histology, Semarang, Indonesia*

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ABSTRACT

Aim of the study: To evaluate the role of micronized purified flavanoid fraction and ethanol *Graptophyllum pictum* extract in the treatment of anal ulcer.
Method: Twenty-eight Wistar rats were randomly allocated into four groups. Groups 2, 3 and 4 the anus were induced with croton oil, but was not induced on group 1. Groups 1 and 2 were treated with normal saline, while groups 3 and 4 were treated with micronized purified flavanoid fraction, and ethanol *G. pictum* extract, respectively. On 9th days blood sample were taken from the retro-orbital region, and Wistar was killed by cervical dislocation under ether anesthesia. The anal canal was resected up 2 cm from anal opening, weighted, photographically taken to measure the percentage of residual ulcer, and then prepared for microscopic examination. Elisa methods were done for superoxide dismutase and malondialdedhyde. The total leukocyte in the anal specimen was counted under 400 magnification power. superoxide dismutase, anal coefficient, and total leukocyte for statistical analysis were using ANOVA and LSD, while malondialdedhyde and percentage of ulcers were using Kruskal–Wallis and Mann–Whitney.
Result: Treatment with ethanol *G. pictum* extract dose of 100 mg/kg BW significantly reduces the percentage of anal ulcer, the edema, leukocyte infiltration, and malondialdedhyde, and increase the superoxide dismutase in comparison without treatment. Treatment with micronized purified flavanoid fraction did not reduce the leukocyte, anal coefficient, and percentage of anal ulcer, only increase malondialdedhyde and decrease superoxide dismutase significantly.

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* *Corresponding author.*
E-mail: iriwanto@gmail.com (I. Riwanto).
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Papel da fração flavonoica purificada micronizada e do extrato etanólico de *Graptophyllum Pictum* em experimento de cicatrização de úlcera anal. Estudo com ratos Wistar

Palavras-chave:
Úlcera anal
FFPM
Extrato etanólico de *Graptophyllum pictum*
MDA
SOD

RESUMO

Objetivo do estudo: Avaliar o papel da Fração Flavonoica Purificada Micronizada e do Extrato Etanólico de *Graptophyllum pictum* no tratamento de úlcera anal.
Método: Vinte e oito ratos Wistar foram randomicamente alocados em quatro grupos. Nos grupos 2, 3 e 4, indução com óleo de cróton foi realizada no ânus, excetuando-se o Grupo 1. Os grupos 1 e 2 foram tratados com solução salina normal, enquanto os grupos 3 e 4 foram tratados com fração flavonoica purificada micronizada e extrato etanólico de *Graptophyllum pictum*, respectivamente. No nono dia, amostras de sangue foram colhidas da região retroorbital, e o rato Wistar sofreu eutanásia por deslocamento cervical sob anestesia com éter. O canal anal foi ressecado até 2 cm da abertura anal, ponderado e fotografado para medir a porcentagem de úlcera residual e, em seguida, preparado para exame microscópico. Os métodos superoxide dismutase e malondialdedhyde do ensaio Elisa foram realizados. A contagem total de leucócitos foi realizada na amostra anal com ampliação de 400 vezes. ANOVA e LSD foram utilizados para a análise estatística de superoxide dismutase, coeficiente anal e número total de leucócitos, enquanto os testes de Kruskal-Wallis e Mann-Whitney foram utilizados para a análise de malondialdedhyde e porcentagem de úlceras.
Resultado: O tratamento com o extrato etanólico de *Graptophyllum pictum* (100 mg/kg de peso corporal) reduz de modo significativo a porcentagem de úlceras anais, o edema, a infiltração de leucócitos e o malondialdedhyde e aumenta a superoxide dismutase, comparado ao não tratamento. O tratamento com a fração flavonoica purificada micronizada não reduziu os leucócitos, o coeficiente anal e a porcentagem de úlceras anais, apenas aumentou o malondialdedhyde e diminuiu significativamente a superoxide dismutase.

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Introduction

To induce the development of hemorrhoids on animal experimental (Wistar) is using croton oil induction on anal.[1,2] Induction of croton oil results in inflammatory and mucosal ulceration of anus.[1] Several animal studies on hemorrhoid treatment were on the anti-inflammatory effect,[1,2] while the healing ulcer effect has never been reported. From a meta-analysis of several RCT, micronized purified flavanoid fraction (MPFF) has been reported effective in reducing hemorrhoid symptoms, such as bleeding, swelling and pain,[3,4] and also reported effective to reduce bleeding and pain postoperatively.[5] Justicia picta or *Graptophyllum pictum* extract (GPE) has also been reported effective in reducing inflammation.[6,7] As MPFF and GPE have anti-inflammatory, it is hypothesized that MPFF and GPE have a potency of speed ulcer healing. Since most traditional medicines come from the plant, the role of ulcer healing from MPFF and GPE should be explored from its anti-inflammatory and antioxidant status.

The primary purpose of the study is to know the role of MPFF and GPE in healing anal ulcers, induced by croton oil. The secondary outcomes of the study are to know the effect on anal edema, total leukocyte infiltration, and the oxidant status.

Materials and methods

It was an experimental study using the Wistar rat, which is induced to suffer hemorrhoids and anal ulcers using croton oil. The anti-inflammatory effects of MPFF and GPE were measure from the edema and total leukocyte count of the Wistar rats anus, while the antioxidant status was measured from serum MDA and SOD. It was difficult to measure the anal edema macroscopically, therefore the anal coefficient, that is the ratio between anal weight in mg to body weight in g, was used.[8] The healing capacity was measure from the percentage of ulcers from anal circumferential at the end of the study.

Ethanol G. pictum extract (EGPE)

G. pictum (GP) is a shrubs plant, member of Acanthaceae family or Justicia picta, which is believed to be native of New Guinea.[7] Nowadays GP can be found in tropical country 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 15–20 °C.[9,10] The

dose of EGPE was 100 mg/kg body weight given twice daily, as already been used by the previous research.[6,11]

Micronized purified flavanoid fraction (MPFF)

The human dose of MPFF in the acute phase of hemorrhoid is 3000 mg/day for adult.[12] Adult body weight is around 50–70 kg, so each kg body weight needs 40–60 mg. According to Nair et al. (2016),[13] to calculate daily dose for the animal is using the equation:

$$\text{Animal equivalent dose (mg/kg)} = \text{human dose (mg/kg)} \times \text{Km ratio.}$$

Km is the correction factor, and for Wistar rat is 6.2. Using human dose of MPFF is 40 mg/kg BW, Rat Equivalent dose is $40 \times 6.2 = 248$ mg/kg BW. Wistar rat body weight is around 200 g; therefore, each Wistar rat will get 248 mg divide by five is 49.6 mg a day. For practical purposes, each Wistar rat gets 50 mg MPFF, divided into two doses. MPFF liquid was processed from Ardium (R/Servier France). Five hundred mg Ardium to be macerated to become powder and dissolve with 20 cc water, so each cc solution contains 25 mg MPFF. Each experimental Wistar rat was given a 2×1 cc MPFF solution.

Croton oil

The croton oil was provided online from Sigma–Aldrich Company, catalog no. C6719-10G. The preparation of croton oil application was deionized water, pyridine, diethyl ether, and 6% croton oil in diethyl ether at a ratio of 1:4:5:10. Following overnight fasts, sterile cotton swabs (4 mm in diameter) immersed in 100 μL croton oil preparations were then inserted into the anus (rectal part, 15 mm from the anal opening) of targeting Wistar mice and kept in the anus for 30 s, once a day for three consecutive days.[1]

Animals

The study used healthy male adult Wistar rats at the age of 10–12 weeks, with the weight around 200 g. The animal will be excluded if, during 7 days, observation appeared to be sick or dying. The Wistar were obtained from the animal house unit of the Lembaga Pengembangan Penelitian Terapan (LPPT) University of Gajahmada, Yogjakarta. The rats were maintained in wire bottom cages at $22 \pm 3\,^{\circ}C$ and 50% to 60% humidity under a 12–12 h light–dark cycle for at least one 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 investigation. The Ethical Committee of the Faculty of Medicine, University of Diponegoro, Dr. Kariadi Hospital, Semarang, Indonesia approved the experimental protocol. Animals 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.[14]

Experimental design

Wistar rats subjects who meet the inclusion criteria were randomly allocated into four different groups. Group 1 was not induced by croton oil and given physiological saline orally (negative control). Groups 2, 3 and 4 were induced by croton oil. Group 2 was given physiological saline (positive control). Group 3 was treated with MPFF, while group 4 was treated with EGPE 100 mg/kg BW. Each of the Wistar was weighted using gram weightier at the beginning of the study. The induction of croton oil was performed once a day for the three consecutive days and the treatment accordingly for the next 5 days. Termination and evaluation of the variables are executed on the 9th day. Blood samples were taken from the retro-orbital sinus. Under ether anesthesia, neck dislocation was done, and the anus containing internal and external sphincter were then resected, with the margin 2 cm wide from the outer border of the anus.[8]

Each anal specimen was weighted with mg weightier, photographically and measured the percentage of the rest of the ulcer. The anal coefficient was measured based on the equation: the anal weight (mg) divided by Wistar body weight (g). The specimens were prepared for microscopic examination. Specimen as thick as 6 μm, stained with Hematoxylin and Eosin to evaluate the leukocyte count. The leukocytes were counted under 400 magnification powers for five fields, and the result was an average of the five fields. The blood was prepared for the examination of serum SOD, MDA using the Elisa method. Animal manipulation and Eliza examination were completely done in LPPT. Leukocyte count of the anal specimen was done in Anatomical Pathology of National Diponegoro Hospital, Faculty of Medicine, Diponegoro University.

Statistical analysis

Since all variables under study were numeric, normal distribution analysis was done. SOD, anal coefficient and total leukocyte were normally distributed, ANOVA, and post hoc LSD were used for statistical analysis. Serum MDA and percentage of ulcers were non-normally distributed; nonparametric Kruskal–Wallis and Mann–Whitney were used to testing the differences.

Result

All experimental Wistar rats were still alive and healthy until the end of the study, and measurement of all studied variables can be done in all Wistar. The mean ± SD weight of Wistar rat in Group 1 was 219.61 ± 26.57 g and significantly higher in comparison with Groups 2, 3 and 4 (173.84 ± 13.37, 173.40 ± 22.33, 177.62 ± 14.59 g) respectively (ANOVA, $p = 0.000$). Therefore for measuring the edema of the anus, did not use the weight of anus, but used anal coefficient. The mean ± SD of the anal coefficient of groups 1, 2, 3 and 4 were respectively 1.88 ± 0.52, 3.13 ± 0.85, 2.64 ± 0.46 and 2.46 ± 0.41 (ANOVA, $p = 0.006$). Fig. 1 showed that after induction of croton oil, the anal coefficient becomes significantly higher (LSD group 1 vs. group 2, $p = 0.001$). MPFF treatment reduced the anal coefficient but not statistically significant (LSD group 2 vs. group 3, $p = 0.132$). EGPE dose of 100 mg significantly reduces the anal index (LSD group 2 vs. group 4, $p = 0.042$). There was no difference between MPFF and EGPE (LSD group 3 vs. group 4, $p = 0.563$).

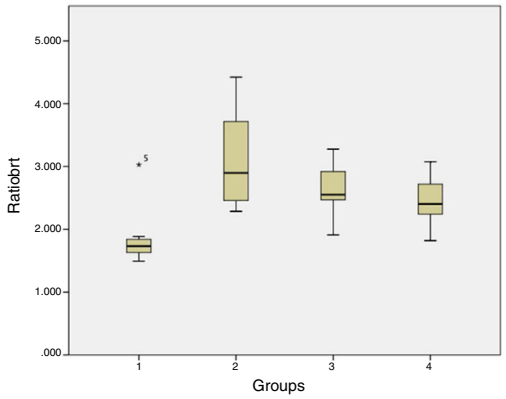

Fig. 1 – Boxplot of a anal coefficient. It is a significant difference among groups (ANOVA, $p = 0.006$). On LSD, group 3 (MPFF) was not significantly different compared to group 2 (positive control) ($p = 0.132$), group 4 (EGPE) was statistically lower in comparison with group 2 ($p = 0.042$).

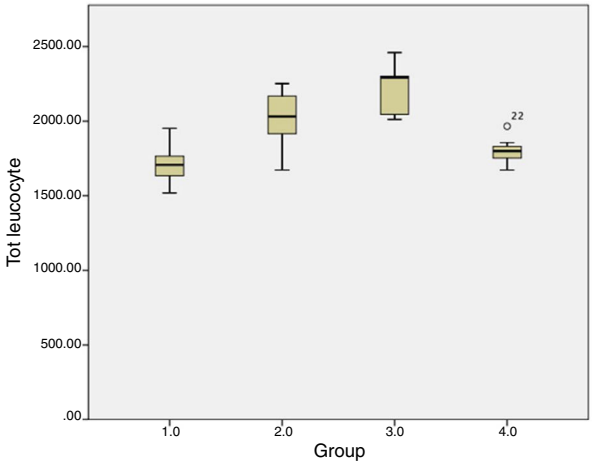

Fig. 2 – Boxplot of total leukocyte at the end of the study. Total leukocytes were a significant difference among groups (ANOVA, $p = 0.000$). Group 3 (MPFF) significantly higher compared to group 2 (LSD, $p = 0.033$). Group 4 (EGPE) was significantly lower compared to group 2 (positive control) (LSD, $p = 0.016$). Group 4 was significantly lower in comparison with group 3 (LSD, $p = 0.000$).

The mean ± SD total leukocyte in the anal specimen on groups 1, 2, 3 and 4 were respectively 1711.00 ± 139.84, 2017.71 ± 200.33, 2208.28 ± 173.01 and 1800.85 ± 96.55 (ANOVA, $p = 0.000$). Induction of croton oil increase of the leukocyte count (group 2 vs. group 1, LSD, $p = 0.001$). Group 3 (MPFF) significantly higher compared to group 2 (positive control) (LSD, $p = 0.033$), while group 4 (EGPE) significantly lower compared to group 2 (LSD, $p = 0.016$). Group 3 vs. group 4 was significantly different (LSD, $p = 0.000$) (Fig. 2).

Fig. 3 shows that without croton oil induction, there was no ulceration (A). Croton oil induction for three days and after followed up for the next 5 days showed complete healing on (B), residual ulcer 40% (C) and residual ulcer 100% (D). There was no ulcer in the group 1, while on the groups 2, 3 and 4 the mean ± SD percentage of residual ulcers was 37.14 ± 32.51, 17.85 ± 16.29 and 8.57 ± 9.88 respectively, Kruskal–Wallis test was $p = 0.003$. Treatment with MPFF reduced the percentage of the ulcer but statistically not significant (group 3 vs. group 2, Mann–Whitney, $p = 0.197$). Treatment with EGPE 100 mg significantly reduces the residual ulcer (group 4 vs. group 2, Mann–Whitney, $p = 0.033$). There was no difference between MPFF and EGPE (group 4 vs. group 3, Mann–Whitney, $p = 0.264$) (Fig. 4).

The mean ± SD of MDA level of the negative control, positive control, MPFF, and EGPE groups were respectively 4394.00 ± 187.88, 4952.53 ± 206.17, 1865.42 ± 113.49 and 935.42 ± 86.50, Kruskal–Wallis test, $p = 0.000$. Induction with croton oil increases the level of MDA level significantly (group 1 vs. group 2, Mann–Whitney, $p = 0.002$). Treatment both with MPFF or EGPE reduce significantly the level of MDA (Mann–Whitney, $p = 0.002$ and $p = 0.002$ respectively) compared to group 2, and EGPE significantly better in reducing MDA in comparison with MPFF (group 4 vs. group 3 Mann–Whitney, $p = 0.002$) (Fig. 5).

The mean ± SD SOD level on groups 1, 2, 3 and 4 were 32.04 ± 4.76, 10.53 ± 6.74, 54.74 ± 2.51, and 70.41 ± 4.14 (ANOVA, $p = 0.000$). On LSD, treatment with MPFF (group 3) and EGPE (group 4) was significantly different in comparison with control positive (group 2) (LSD, $p = 0.000$ and $p = 0.000$ respectively) and EGPE treatment better significantly than MPFF treatment ($p = 0.000$) (Fig. 6).

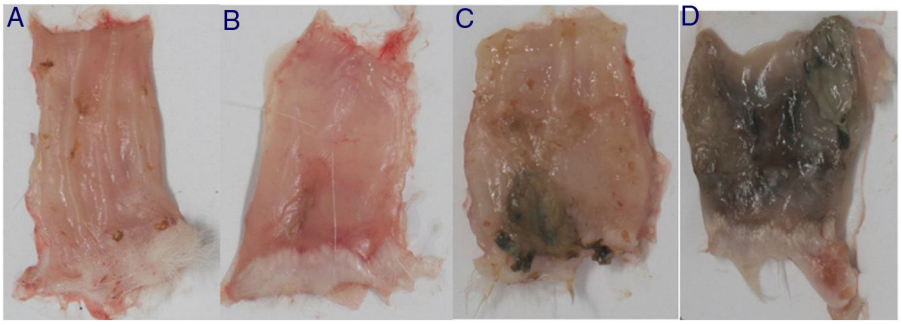

Fig. 3 – Longitudinal transection of the Wistar anus. (A) Normal anal mucosa, without croton oil induction; (B) ulcer heal 100% after croton oil induction; (C) ulcer around 40% of anal circumference; (D) ulcer on all anal circumference (100%).

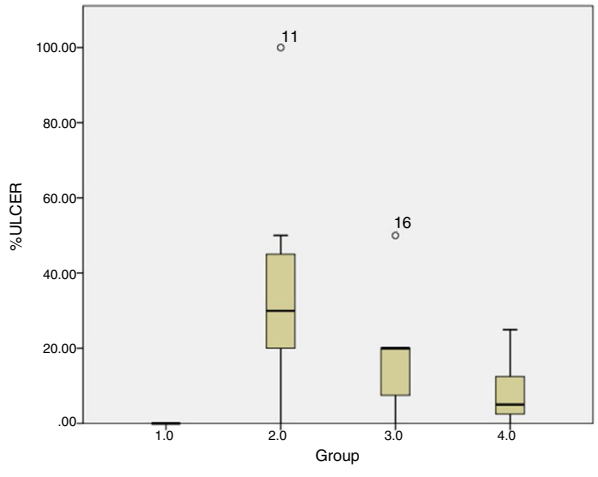

Fig. 4 – Boxplot of the percentage of residual ulcers at the end of the study. It is a significant difference among groups (Kruskal–Wallis test, $p = 0.003$). On Mann–Whitney, group 3 (MPFF) was not significantly different compared to group 2 (positive control) ($p = 0.197$), group 4 (EGPE) was statistically lower in comparison with group 2 ($p = 0.033$).

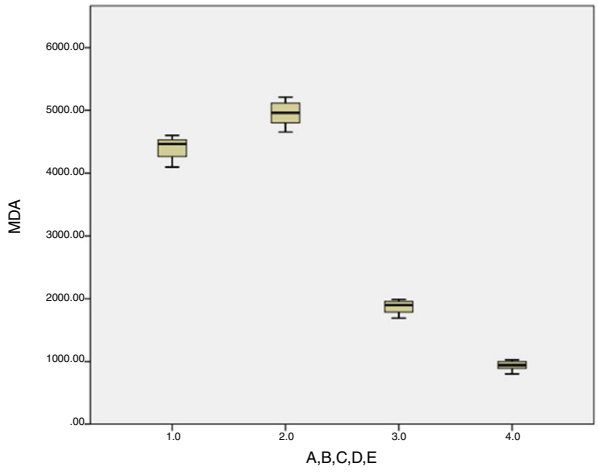

Fig. 5 – Box-plot of MDA level at the end of the study. It is significantly different among groups (Kruskal–Wallis test, $p = 0.000$). On Mann–Whitney test, group 3 (MPFF) and group 4 (EGPE) were significantly different compared to group 2 (positive control) ($p = 0.002$ and $p = 0.002$ respectively), group 4 was statistically lower in comparison with group 3 ($p = 0.002$).

Discussion

Croton oil is an irritant that may cause inflammation, necrosis, and ulceration.[1] In this study, induction with croton oil was up to 15 mm from the anal opening of targeting Wistar mice and kept in the anus for 30 s, once a day for three consecutive days. Irritation with croton oil induce the necrosis of the anal mucosa, and serial anal induction will result in mucosal ulceration. Therefore, it was believed by this method; ulceration has happened in all Wistar anus. Inflammation is a response

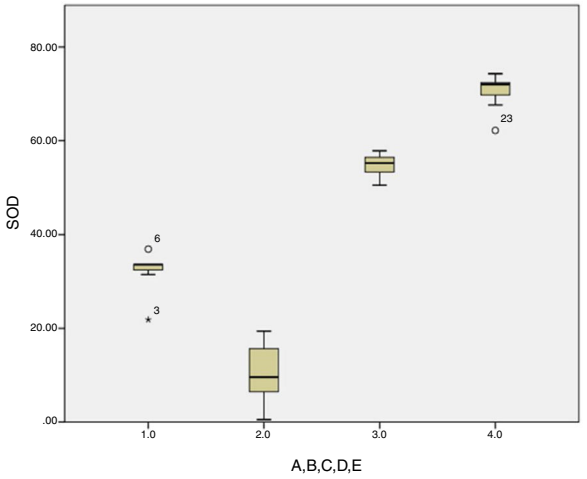

Fig. 6 – Boxplot of SOD level at the end of the study. It is significantly different among groups (ANOVA, $p = 0.000$). Group 3 (MPFF) and group 4 (EGPE) were significantly higher compared to group 2 (positive control) (LSD, $p = 0.000$ and $p = 0.000$ respectively), group 4 was statistically higher compared to group 3 (LSD, $p = 0.002$).

of the host to infection or tissue damage in the purpose of tissue hemostasis. Uncontrolled inflammation may lead to tissue damage, delayed recovery, loss of organ functions, and chronic inflammation.[15,16] Tissue necrosis release alarmin,[17] or damage associated molecular pattern (DAMP),[18] that through Toll-like receptor 4 activate NF-kB to produce proinflammatory cytokine, result in a vascular leak, tissue edema and increasing the leukocyte in the tissue.[17] The result of this study was in accord with the above concept. Induction with croton oil increase significantly anal coefficient, as representative of tissue edema, and total leukocyte count. Inflammation reaction is important for initiating tissue healing since tissue necrosis and may be bacterial contamination should be eradicated first.[19] Neutrophils initially and eventually monocyte is moving from intravascular to the tissue to eradicate the necrotic tissue and bacteria. Activated monocyte, macrophage, initially said as M1 function as phagocyte of the rest of necrotic tissue and bacteria and also the dead of neutrophil.[19]

This study showed that treatment with EGPE 100 mg results in resolving the ulcer, decreasing the anal coefficient and total leukocyte better than the positive control group, which means that EGPE speed the ulcer healing through its potent anti-inflammation. Meanwhile, MPFF was not significantly reduced the ulcer healing, decreasing leukocyte, and anal coefficient. The relationship between the resolving of inflammation and wound healing has already been known well. Homeostasis is recovered after production pro-resolving mediator that act on specific receptor targets to (1) shutdown the recruitment of polymorphonuclear leukocyte, (2) counteract signaling pathways associated with leukocyte survival to promote apoptosis, (3) activate the clearance of apoptosis cells (by macrophages), (4) macrophages reprograming from M1 (pro-inflammatory) to M2 (pro-resolving phenotype).[16] M1 macrophages are induced to develop into a novel M2-like phenotype via an IL-4/IL-13 independent manner.[19] Aborted neutrophil recruitment is the

initial step to achieved tissue hemostasis, followed by apoptosis of the rest of neutrophil and clearance by macrophages.[16] M2 consisted M2a, M2b, and M2c and M2c has roles in matrix deposition and tissue remodeling.[20] The study showed that M2 macrophages express of anti-inflammatory mediators, they are IL-1R antagonist, decoy IL-1 receptor Type II and IL-10, and production of TGF-beta1, VEGF and IGF-1.[19] TGF-ß is a cytokine important for inflammation resolution, has a role in epithelial restitution and fibrosis. TGF-ß acts as a potent negative regulator to mucosal inflammation.[16] VEGF induces new vascularization important for wound healing.[19]

During the inflammatory process, neutrophil and macrophage produce oxidant that important to kill bacteria, but excessive production results in oxidative stress.[18] The marker for oxidative stress is MDA.[1,21] MDA is originated under stress conditions as an end product generated by decomposition of arachidonic acid and larger PUFAs, that has a high capacity of reaction with multiple biomolecules such as proteins or DNA that lead to the formation of adducts. MDA is strongly reactive toward nucleophiles, such as basic amino acid residues (i.e., lysine, histidine, or guanine).[21] In this study showed that after induction with croton oil, the MDA level was increased significantly. As a compensation body react the oxidant by producing the endogen antioxidant, such as SOD. These results were in accordance with Gurel et al.'s result (2013).[1] The body response to produce antioxidant endogen depends on the condition of the body.[22] This study showed that the response of the rat to high MDA by producing SOD was not so good. The SOD level of the croton induced rat was significantly lover in the positive control group (group 2). However, after treatment with both MPFF and EGPE significantly reduce the MDA and increase the SOD, and EGPE better than MPFF. This result showed that MPFF and EGPE have antioxidant properties. SOD is an enzyme that repairs cells and reduces the damage caused by superoxide, a common free radical in the body.[1] SOD is required for postnatal angiogenesis, and SOD plays an essential role in reparative neovascularization in response to ischemic injury by protecting ischemic tissues from an overproduction of O_2^{*-}.[22]

The mechanism of how EGPE has anti-inflammatory properties is not known yet. Some natural products are reported that may block the formation of TLR4/MD2 complex such us curcumin from Curcuma longa, sulforaphane, and iberin from cruciferous vegetables, xanthohumol from hops and beer and celastrol from *Tripterygium wilfordii*. Among them, Xanthohumol is a chalcone type flavonoid of *Humulus lupus*. Toll-like receptor 4 (TLR4) is a member of TLR as an innate immune response receptor that has an important role in acute inflammations by amplifying the proinflammatory response. Activators of TLR4 are bacteria, fungal, viral infection, and also molecules released by injured cells and tissues (DAMP's, danger-associated molecular patterns). TLR4 becomes active in the form of the TLR4/MD-2 complex.[23] Whether flavonoid within EGPE may block the formation of TLR4/MD2 is not known yet, further study should be planned.

The result of this study showed that EGPE better than MPFF is not easy to be explained. The possible explanation is the difference in its bioactive contents. MPFF contents are 90% diosmin and 10% hesperidin both are purified flavonoids.[24] The contents of ethanol extract of *G. pictum* are more complex; they are alkaloids, glycoside, flavonoids, saponins and tannin while using petroleum ether extracts the contents are glycoside and steroids. The inorganic components of EGPE are calcium, iron, sulfate, phosphate, and chloride.[25]

As conclusion EGPE speed the anal ulcer healing after croton oil induction, better than MPFF, through its anti-inflammatory and antioxidant property. Further studies are needed to know the mechanism of anti-inflammatory and antioxidant of EGPE, especially regarding the possibility of blocking the TLR4.

Conflicts of interest

The authors declare no conflicts of interest.

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