*Apium graveolens* Herbs Ethanolic-Extract Improve Acetic Acid–Induced Colitis Condition in Rats

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**ABSTRACT**

Colitis is a growing gastrointestinal inflammation condition that affect individual all around the globe. No specific treatment for colitis condition is available at the moment and this disease can develop into colorectal cancer. Phytochemical in *Apium graveolens* (AG) are potential as anti-inflammation and antioxidant. This study aimed to investigate the potency of AG ethanolic extract (EES) in improving acetic acid (AA) – induced colitis in rats. Rats with colitis induced by acetic acid (4%) were treated with 5-aminosalicylic acid (5-ASA) as positive control, and several concentrations (100, 300, and 900 mg/kg) of EES. Colitis severity was determined by scoring of macroscopic condition including body weight, colon, and faeces condition. In addition, colon tissues conditions were observed with haematoxylin-eosin staining. Colon permeability is determined by measuring urine glucose level. The observation of the change in *Enterobacteriaceae* population is investigated using PCR. *Apium graveolens* ethanolic-extract shows highest improvement of colitis condition at concentration of 900 mg/kg, based on macroscopic observation and disease severity index. At this dose, no blood existed on the faeces and the faeces consistency is solid. Group receiving lower dose of EES still shows blood existence and less solid faeces. The EES dose dependently return the population of *Enterobacteriaceae* which increase upon AA treatment. *Apium graveolens* is potential to be use as preventive or treatment for colitis.

**Keywords:** *Apium graveolens*, ethanolic-extract, colitis, *Enterobacteriaceae*, inflammation

**INTRODUCTION**

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the intestine that include Crohn’s disease (CD) and ulcerative colitis (UC) (Alatab *et al*., 2020). This disease affected around 6.8 million individuals in 2017 globally (Alatab *et al*. 2020). The incidence and prevalence of IBD in the worlds is continues to grow (Alatab *et al*., 2020; Magro *et al*., 2017). Etiology of UC is still unclear, and the curative therapy is still not available (Magro *et al.*, 2017). Many factors contribute to UC, including oxidative stress, immune disorder, gut-microbiota balance disorder, genetic factor, environmental condition and food intake (Jazayeri *et al.*, 2017; Magro *et al.*, 2017; Nascimento *et al.*, 2020). Without appropriate treatment, UC can develop into more life-threatening disease such as colorectal cancer (Chang *et al*., 2018).

Inflammatory bowel disease attacked wide age range, from childhood to late adult (Alatab *et al*., 2020). Most UC patient is undergo pharmacological therapy using 5-aminosalicylates, corticosteroids, and immuno-suppressants. However, cushing’s syndrome as therapy adverse effect is commonly occurs and should be considered for therapy using above mentioned agents (Butter *et al.*, 2018).
Food nutritional component were reported to modulate gut inflammation (Uranga et al., 2016). Phytochemicals in food (amino acid, peptide, fatty acid, vitamin, antioxidant, and food fiber) might affect gut-microbiota balance, gut permeability, protein secretion and gut protection to inflammation (Jazayeri et al., 2017; Nascimento et al., 2020; Uranga et al., 2016).

*Apium graveolens* herbs is commonly consumed as food ingredients, and are able to maintained mood and cognitive function, gastrointestinal health, and minimize inflammation condition (Kooti et al., 2014). *Apium graveolens* also have phytochemical constituent (flavonoid and polyphenol) that are potential as anti-inflammatory and antioxidant (Liu et al., 2020). Suppression of pro-inflammatory cytokine i.e., IL-1β and TNF-α after the administration of AG aqueous extract were reported in hyperuricemia mice. In addition, the same extract was stimulated IL-10 secretion (Soliman et al., 2020).

Previous report showed the methanolic- and hexane-extract of AG seeds improve colitis condition in acetic acid-induced rats (Minaian et al., 2021). This study investigated the potency of AG herbs ethanolic extract (EES) in improving colitis condition. In addition, inflammation condition of the colon, the investigation on the change of gut microbiota population.

**MATERIALS AND METHODS**

*Apium graveolens* powder was purchased from Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (Karanganyar, Indonesia). Apigenin, ethanol 96% and 5-aminosalicylic acid (5-ASA) were obtained from Sigma-Aldrich (Darmstadt, Germany).

**Apium graveolens** extraction

Prior to extraction with ethanol 70%, dry AG powder was sieved with 40 mesh sieves. The extraction process is following Ultrasonic Assisted Extraction (UAE) method as described by (Wei & Yang, 2017). In brief, 300 g of AG powder was immersed in seven parts of ethanol 70%. Then sonicate (230-volt, 360 watt, 1.5 ampere, 50 Hz) for 30 min at room temperature (RT). Evaporate extraction solution at 50°C until obtaining concentrated AG ethanolic-extract (EES).

Extract characterization was carried out with thin layer chromatography (TLC). Ten milligrams of the EES were dissolved in 1 mL of ethanol. Ten microliters of the solution were applied to a 60 F 254 silica gel plate, along with apigenin standard. The development of the TLC plate was performed using glass tank which had been pre-saturated with a mixture of toluene:ethyl-acetate:formic acid (7:2.5:0.5) as mobile phase. After ascending development, the plate was air dried and the compounds were visualized by UV light at 366 nm after sprayed with AlCl₃ reagent.

Total flavonoid content was determined with AlCl₃, following method described by (Chang et al., 2020). Apigenin was used as markers and was dissolved to make standard concentration of 25, 50 and 100 μg/mL. Each of these standard solutions (0.5 mL) were mixed with 1.5 mL of 96% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. Incubate for 30 min at RT, and read the absorbance at 415 nm. The amount of 10% aluminum chloride was substituted by the same amount of distilled water for blank (Chang et al., 2020).

**Animal maintenance and treatment**

This work is performed after obtaining ethical clearance from The Ethical Committee of Laboratorium Penelitian dan Pengujian Terpadu Universitas Gadjah Mada (LPPT UGM) with reference number 0006/04/LPPT/I/2021. Thirty five male wistar rats (4 – 6 months old, 250-350 g) were randomly divided into seven groups (5 rats each groups) as follows: 1) group receiving intragastric administration of 0.25% CMC-Na without colitis induction (normal control); 2) group receiving intragastric administration of 0.25% CMC-Na with colitis induction with intrarectal administration of 4% acetic acid; 3) group receiving intragastric administration of 0.25% CMC-Na with colitis induction with 4% acetic acid, and 100 mg/kg body weight (further will only written mg/kg) of 5-aminosalicylic acid (5-ASA) given via drinking water; 4) group receiving intragastric administration of 0.25% CMC-Na with colitis induction with 4% acetic acid, and 100 mg/kg of EES (further will only written mg/kg) of 5-aminosalicylic acid (5-ASA) given via drinking water; 5) group receiving intragastric administration of 0.25% CMC-Na with colitis induction with 4% acetic acid, and 100 mg/kg of EES; 6) group receiving intragastric administration of 0.25% CMC-Na with colitis induction with 4% AA, and 300 mg/kg of EES; 7) group receiving intragastric administration of 0.25% CMC-Na with colitis induction with 4% AA, and 300 mg/kg of EES; 8) group receiving intragastric administration of 0.25% CMC-Na with colitis induction with 4% AA, and 1000 mg/kg of AG bulk powder suspension. Colitis is triggered with single per rectal administration of 4% AA. Five hundred microliter of AA is per rectally injected with 1 mL syringe and 22 G canula, following per rectal application previously.
Colitis severity scoring and colon histology

Body weight, feces consistency and blood existence were scored following the scoring method (Supplementary Table I) explained elsewhere to obtain colitis severity condition (Tian et al., 2016). The animals were sacrificed with intraperitoneal injection of ketamine-xylazine (0.3 ml:0.15 ml per 100 g rats). Prior to fixation, the colon weight, length, and diameter were measured. In addition to colon, spleen was also collected and the weight is measured. Colons were fixed with 10% of Neutral buffered formalin solution (Sigma, Darmstadt, Germany) and made to paraffin blocks. Tissue blocks were sliced to thickness of 4 μm, then stained with haematoxylin-eosin (HE) to evaluate the sign of inflammation and tissue damage. The microscopic conditions were then scored according to Supplementary Table II following previous study by (Minaiyan et al., 2014). A score of “0” means the condition is normal (as shown in mice without treatments), and “3” represents the most severe form of damage.

Urine glucose measurement

The rats fasted overnight prior to sucrose administration. Sucrose was administered orally at a dose of 750 mg/kg in a saline solution. Four hours following sucrose administration, urine was collected (Ghattamaneni, 2019) and stored at -20 °C until the glucose concentration determination. Glucose concentration was measured using an enzymatic photometric test containing a glucose oxidase enzyme (Glucose GOD-PAP, Neudorf, Austria) using 10 μL of samples. Each sample were mixed with 1 mL of reagent and incubated at RT for 20 min. The solution absorbance was measured at 546 nm.

Enterobacteriaceae detection

Total gut commensal bacterial was isolated and purified from rat feces using FavorPrep™ Stool DNA Isolation Mini Kit (Favorgen Biotech Corp, Ping-Tung, Taiwan) following manufacturer protocol. Two hundred ng of the DNA sample was mix with MyTag HS Red Mix (Meridian BioScience, USA) for PCR. DNA amplification was performed with two pairs of primers, each pair for detection of 16s rRNA and Enterobacteriaceae gene. Forward and reverse primer for Enterobacteriaceae amplification is 5′ CAG GTC GTC ACG GTA ACA AG 3′ and 5′ GTG GGT CAT TTT CAG CAT GTA C 3′, respectively. Forward and reverse primer for 16s rRNA are 5′ ACT ACG TGC CAG CAG CC 3′ and 5′ GGA CTA CCA GGG TAT CTA ATC C 3′, respectively (Fazzeli et al., 2012). The PCR were proceeded for 30 cycles with the conditions as follows: initial denaturation at 95 °C (1 min), denaturation at 95 °C (15 s), annealing at 55 °C (15 s), extension at 72 °C 10 s, and final extension at 72 °C (1 min). The PCR product was then run on 0.8% agarose gel electrophoresis, in a Tris-acetic acid- EDTA (TAE) buffer. Amplification band is detected with a gel red detection system (GelRed, Nucleic Acid Gel Stain, Biotium).

Statistical analysis

All quantitative data are presented as Mean ± SD of three replication of experiment or other is state. The significance between groups were tested using ANOVA with Tukey’s test as post hoc analysis using Graph Pad Prism 9.0. The data are statistically significance when p value < 0.05 (confidence level 95%).

RESULTS AND DISCUSSION

Apium graveolens extraction

Total EES obtained was 65.02 g with a total rendemen of 21.67%, which has 10.51 ± 0.34 % moisture content (loss on drying). The thin layer chromatogram of the EES showed a detectable amount of a compound that have an identic profile i.e., 0.31 retention factor (Rf) and green blue colour, with standard apigenin (Supplementary Figure 1). Determination of total flavonoid content by colorimetric method shows the EES contained total flavonoid 9.89 ± 0.42 % w/w equivalent apigenin.

Apium graveolens ethanolic-extract improve colon macroscopic and microscopic conditions

The body weight of rats was not changed upon different treatment. The administration of EES clearly showed the improvement of colon morphological condition in AA – treated rats. The colon weight is decrease in the increase of EES concentration. The colon weight in rat group treated with 900 mg/kg EES is 2.33 ± 0.12 g, which is comparable with colon weight in rat without AA treatment i.e., 1.38 ± 0.02 g (Figure 1B). This weight is far lower compare to colon weight in rat receiving AA only, which weighted 4.07 ± 0.71 g (Figure 1B). The group receiving drugs that often used to treat colitis, 5-ASA, shows colon weight of 2.21 ± 0.19 g.
The lowest-dose of EES shows the shortest colon length compare to other EES-treated rats. This group shows 13.00 ± 0.61 cm of colon length, slightly higher than colon length of group receiving only AA (Figure 1C). However, this still shorter compare to the length of colon in untreated group (16.30 ± 0.44 cm) and group receiving 5-ASA (15.00 ± 0.79 cm). It appears that the variation in EES dose is not affected the length of colon. The colon diameter of rat with AA is contrastly higher (2.48 ± 0.31 cm) compare to other groups (< 1.5 cm) (Figure 1A). The EES did not affect spleen weight (Figure 1D).

Hematoxylin-eosin staining of colon tissues showed no clear different sign of inflammation between groups as expected by colon macroscopic condition. No clear different in mucous, crypt, and muscle thickness could be observed between groups, especially that receiving AA- and EES-treatment. Only goblet cells population between colitis mice and untreated/normal control showed clearer mark of inflammation and non-
inflammation condition. In addition, clear image of inflammatory cell infiltration could not be observed in the tissue section image (Figure 2). In contrast, macroscopic image of colon shows clear different between treatment groups. In control normal, the colon is clean, short in length and diameter. Group receiving only AA shows intense red color and increase in colon diameter (Figure 1A, 3B). Group receiving AA and EES still show redness in the colon but in lower intensity that that in AA-only group (Figure 3D-F). The increase in colon diameter is still observable in group receiving 100 mg/kg EES, but not in the other group (Figure 1A, 3D).

Figure 3. Effect of *A. graveolens* ethanolic-extract (EES) on macroscopic sign of inflammation. A – F subsequently are: normal control (A), acetic acid-treated group (B), 5-ASA-receiving group (C), 100 mg/kg EES-receiving group (D), 300 mg/kg EES-receiving group (E), and 900 mg/kg EES-receiving group (F). Colon sample were taken from single rats within group as a representative.

**Apium graveolens** ethanolic-extract improve colitis condition

All groups treated with AA shows blood in feces after 5 days of AA injection. The blood in feces is continuously diminished in rat with AA treatment where it reached the lowest blood content in feces at day tenth (Figure 4C). The groups with EES administration showed faster depletion of blood existence in feces. The highest EES concentration (900 mg/kg) showed the fastest blood disappeared in feces. This group had no blood in the feces after three days administration of the EES (day 8 after AA injection), which is comparable with 5-ASA group (Figure 4C). Whereas other groups showed complete elimination of blood feces after 4 days EES administration (Figure 4C).

The group treated with 900 mg/kg of EES also showed the highest improvement in feces consistency (Figure 4D). The feces consistency of rat receiving EES at this concentration is comparable to that consistency of group receiving 5-ASA. The EES could not increase body weight to the level of untreated-rat body weight. However, the 5-ASA also failed to increase the body weight of rats (Figure 4B). By combining the existence of blood in feces, feces consistency, and body weight data, the colitis development condition could be concluded. In general, colitis severity was suppressed by the administration of EES. Even though it could not as potent as 5-ASA, high dose of EES is closely suppressed colitis development to the level that showed by 5-ASA (Figure 4A).

**Apium graveolens** ethanolic-extract restore urine glucose concentration

Urine glucose concentration can be used to indicate colon permeability condition. The urine glucose concentration is increase ~5 folds from 5.40 ± 0.25 mg/dL in control group to 24.40 ± 0.37 mg/dL in AA-treated group. The administration of EES 100 mg/kg, 300 mg/kg, and 900 mg/kg decrease the urine glucose level to 15.60 ± 0.25, 14.37 ± 0.17 and 12.30 ± 0.16 mg/dL, respectively (Figure 5). The treatment with EES could not completely restore urine glucose concentration to normal condition.

**Apium graveolens** ethanolic-extract restore *Enterobacteriaceae* population.

Group with AA treatment showed a thicker *Enterobacteriaceae* band compare to group of control (Figure 6). The treatment of the rats with various EES concentration dose-dependently decrease the thickness of the amplification bands. These indicates the increase of *Enterobacteriaceae* in AA-induced colitis model could be suppressed by the administration of EES. However, the EES even at the highest concentration used (900 mg/kg) is failed to recover the *Enterobacteriaceae* population back to normal or to the level that showed by 5-ASA treatment (Figure 6).
**Figure 4.** Effect of *A. graveolens* ethanolic-extract (EES) on colitis activity index. *Apium graveolens* ethanolic-extract attenuate colitis activity index (A) that include three parameters i.e., body weight (B), the existence of blood in the faecal (C), and faecal consistency (D). The data are presented as mean ± SD of the experiment (n = 5). (*) mark the significant different vs group only receiving 4% of AA. No significant different observed between different EES doses. Significant difference between group treatment vs normal control were not marked to provide a clear and easy to understand figure.

**Figure 5.** Effect of *A. graveolens* ethanolic-extract (EES) on rat urine glucose concentration. The data are presented as mean ± SD of the experiment (n = 3). (*) mark the significant different between groups with p value < 0.05. AA 4% = 4% acetic acid; 5-ASA = 5-aminosalicylic acid.
colitis (Li et al., 2019; Minaiyan et al., 2021; Sukketsiri et al., 2016). As minimum as 200 mg/kg hexane-extract and 400 mg/kg methanol-extract of AG seeds were shown to reduce colitis severity and myeloperoxidase (MPO) production in colitis wistar-rat induced by AA 4% (Minaiyan et al., 2021). Apium graveolens seed aqueous-extract and oil-extract reduced the IL-1β and TNF-α levels, and increase IL-10 in arthritis mice (Li et al., 2019). In this study the extract concentration used is lower than that described by Minaiyan group (Minaiyan et al., 2021) as we assumed that this the total active compound obtained by UAE is higher per mg sample. In addition, as low as 200 mg/kg AG seed aqueous-extracts was reported to have anti-inflammatory activities gout (Li et al., 2019).

Colitis induced the change in colon macroscopic condition. Inflamed colon prone to have more weight, and shorter in length compare to non-inflamed colon (Fatani et al., 2016; Jeengar et al., 2017; Minaiyan et al., 2021). Present study confirmed these colon appearances. This study also observed the increased of colon diameter, particularly in the inflamed area. Apium graveolens ethanolic-extract returned all of these macroscopic conditions closed to normal condition. The presence of apigenin, one of the major constituents in AG, in EES produce in this study could responsible for these results. Apigenin was reported to inhibited the colonic inflammation (Gentile et al., 2018; Radulovic et al., 2018).

In addition to colon macroscopic condition, this study also investigated the macroscopic condition of the spleen. Spleen size increase in several diseases i.e., immune related disease, infection and several malignancies (Chapman et al., 2021). As the etiology of colitis is still unclear, the investigation of spleen condition could provide information about the effect of colon inflammation to the lymphocyte homeostasis, especially in the spleen. As the spleen is involved in the immune responses, including inflammation, this secondary lymphoid organ could be affected by the inflammation in the colon. However, this experiment shows that no observable spleen weight different between groups. This could be understood as the increase of spleen size might not only caused by the increase of lymphocytes number or other spleen resides cells, but also the extracellular fluid in spleen. Study in human showed that the size of spleen was increased in Crohn diseases, but not in ulcerative colitis (Kawashima et al., 2022).

The UAE method has been shown to produce higher phytochemical yield and content than the reflux and maceration methods (Chua, 2013). The UAE method employs sonochemistry and mechanical effects induced by ultrasonic cavitation bubbles. High ultrasonic waves might result in a pressure that trigger the formation of cavitation bubbles which allowed the solvent to penetrate extensively into the plant material, rupture the plant cell walls, and simultaneously promotes the release of bioactive compounds (Chua, 2013). The UAE method is applicable for wide variety of samples and have several benefits including the decrease in extraction and processing time (Chemat et al., 2017). Direct comparison of the apigenin obtained from this study with other method could not be performed at present, as no completely identical method is used between studies. For example, Derouich et al., use rutin equivalent to determine the flavonoid content in Apium graveolens extract (Derouich et al., 2020), but in this study apigenin was used. In addition, this study also not aimed to focus on the comparison of the extraction method.

Apium graveolens improved various inflammation condition including arthritis and inflammation.
Feces of individuals with colitis were loose, and in more severe condition, blood clot can be observed (Jeengar et al., 2017; Tian et al., 2016). Feces consistency, the presence of blood in feces, and urine glucose concentration indicate the disturbance on colon integrity and normal function. *Apium graveolens ethanolic-extract* improved colon function as barrier as marked by the recovery of sucrose level in group receiving EES compare to group receiving AA only. Healthy colon should re-absorb water only, but not glucose. *Apium graveolens ethanolic-extract* return the blood vessel permeability, where the blood presence is decrease after EES treatment. Feces consistency also not loose indicating the ability of colon to reabsorb water is recovered. No significant effect of EES on body weight recovery was observed.

Limitation of this study is no clear histological image of colon tissue were obtained. Only goblet cells and inflammatory cell could be clearly observed from the histological image. The unclear image of tissue after HE staining caused tissue condition scoring could not completely made. The inflamed colon should show mucus depletion, epithelium destruction, edema, erosion of the colonic crypts and infiltration of inflammatory cells (Hanci et al., 2015; Minaian et al., 2021; Radulovic et al., 2018). Macroscopically, colon condition was clearly improved. However, microscopically, improvement of mucus content, epithelium and crypts condition, and inflammatory cell infiltration was not obvious. One important rationalization of the histopathology result was because HE staining of the tissue samples were not made in the same section of colon such as ascending, descending, or transverse colon (Kahai et al., 2021). The area that shows inflamed condition macroscopically (i.e., redness or large diameter of the colon) were taken for tissue paraffin block, even though they located in different part of colon. This could contribute to the scoring is not applicable for tissue section. The tissue for making paraffin block should be taken from the same/close part of colon between groups. However, because the same part of colon between group are macroscopically different, this close part is not taken for making tissue section. It is assumed that this part will definitely give significant microscopic image. This study is interested in finding any different in remaining inflamed condition between group. However, this cause negative results.

Inflammation in colon caused the changed in gut microbiota population (Baldelli et al., 2021; Radulovic et al., 2018; Sovran et al., 2018). In particular *Enterobacteriaceae* population was reported to increase in colitis condition (Baldelli et al., 2021). *Enterobacteriaceae* is needed for colitis development. The presence of *Enterobacteriaceae* is essential for the effect of other microbiota i.e., fungi on gut the inflammation, possibly through their positive influence on fungal colonization (Sovran et al., 2018). Present study confirmed the increase of *Enterobacteriaceae* in colitic rat with PCR method. Polymerase chain reaction is not a method of choice as its less sensitive and selective compare to qualitative/real time-PCR or DNA sequencing that are often used for determination of the existence of bacterial taxa or families in samples. However, this method provided minimum information about the microbiota population, and is proved to be in line with available reports (Baldelli et al., 2021; Radulovic et al., 2018; Sovran et al., 2018). Suppression of *Enterobacteriaceae* population by EES showed in this study could possibly account for the existence of apigenin in the EES. Apigenin could recovered the bacterial diversity in the feces of colitis mice in the mechanism that dependent on an intact Nlrp6 signaling pathway (Radulovic et al., 2018).

Taken together, this study showed that apigenin-containing EES improve colon inflammation in AA-induced colitis model in rats. This EES also recovered gut microbiota population close to normal condition. This EES has a potency to be developed as supporting supplement to prevent and treat colitis.

**CONCLUSION**

*Apium graveolens*-ethanolic extract improve colon inflammation and recover gut permeability in acetic-acid induced colitic in rats. The extract also recover gut *Enterobacteriaceae* population that increase in colitic rats. *Apium graveolens*-ethanolic extract have a potency to be developed as co-treatment for colitis therapy.

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CONFLICT OF INTEREST

The author have no conflict of interest to declare. Above funding bodies have no conflict of interest with present studies.

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