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Research Article
Anti-inflammatory Activity of *Etlingera elatior* (Jack) R.M. Smith Flower on Gastric Ulceration-induced Wistar Rats

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Abstract

Background and Objective: *Etlingera elatior* (Jack) R.M. Smith rhizome, which has been traditionally used to reduce stomach discomfort, was reported to possess anti-inflammatory activity, however, there is a lack of such a study on the flower. **Materials and Methods:** To investigate the anti-inflammatory activity of the *E. elatior* flower extract on gastric ulceration-induced Wistar rats. The Wistar rats were divided into 6 groups. Group 1 was the normal control, group 2 was the negative control (Arabic gum suspension 2%), group 3 was the positive control (quercetin), group 4-6 were treated with *E. elatior* flower extract dose of 500, 1000 and 2000 mg kg^{-1} of b.wt., respectively. The rats were conditioned to gastric ulceration. The stomach weight, microscopic and macroscopic evaluation of gastric mucosal damage was examined. Subsequently, the nuclear factor-kappaB-p65 (NF-kappaB-p65) expression in the fundus was Western-blotted by employing β-actin and GAPDH as the loading controls. **Results:** *Etlingera elatior* flower extract dose of 1000 mg kg^{-1} b.wt., reduces the ulceration index and the infiltration of inflammatory cells. Western blot analysis showed inhibition of NF-kappaB-p65 expression by *E. elatior* flower extract dose of 1000 mg kg^{-1} of b.wt. **Conclusion:** *Etlingera elatior* flower might possess anti-inflammatory activity by downregulating the expression of NF-kappaB-p65 in the fundus of gastric ulceration-induced Wistar rats.

Key words: Anti-inflammation, *Etlingera elatior*, gastric ulceration, nuclear factor-kappaB-p65

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Gastric inflammation is attributed to ulcer formation accompanied by epithelial cells apoptosis, vascular reactions development and inflammatory cell recruitment followed by intraepithelial neutrophils infiltration[1,2]. Eventually, the inflammatory cells will intensively generate free radicals, growth factors and NF-kappaB-dependent pro-inflammatory cytokines i.e., inducible Nitric Oxide Synthase (iNOS), interferon-gamma (IFN-γ, interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-8 (IL-8), within the gastric tissue[3,4]. The strong acidic condition in the gastric (pH 1-3) could induce the expression of NF-kappaB in esophageal cell lines[5]. Furthermore, this physiological low pH also triggers the expression of the downstream protein IL-8[6]. Overall, an inflammatory process is initiated and regulated by chemical signaling and involves a series of molecular reactions and cellular activity either on the physiological or pathological processes[7].

Etlingera elatior (Jack.) R.M. Smith (Zingiberaceae) has been traditionally used to reduce stomach discomfort[8]. Secondary metabolites such as; phenolics and flavonoids[9], terpenoids, steroids, tannins, glycosides, saponins and flavonoids[10] have been identified in the flower. Flavonoids contained in *E. elatior* are kaempferol, quercetin and myricetin[11]. The leaves of *E. elatior* consisted of quercetin 3-glucoside, quercetin 3-glucuronide, quercetin 3-rhamnoside and kaempferol 3-glucuronide, while the flowers and stems produced kaempferol-3-O-glucoside, kaempferol and quercetin[12,13].

Etlingera elatior rhizome has been reported in possessing a scavenging effect against free radicals by reducing the violet 2,2-diphenyl-1-picrylhydrazyl (DPPH) to the yellow DPPH-H. The rhizome extract potentially inhibits Nitric Oxide (NO) production in RAW264.7 cell-line (IC_{50} of 19.36 μg mL^{-1}), down-regulates the expression of p38, ERK1/2, Akt, P-p38, P- ERK1/and P-Akt in B16 melanoma cells[14]. However, not any reported on the anti-inflammatory activity of the flower. This study discovers the anti-inflammatory activity of *E. elatior* flower that can be beneficial for reducing gastric ulceration. It will help the researcher to uncover the critical areas of inflammation occurs in the stomach that many researchers were not able to explore. Thus, a new theory on plant-based anti-inflammatory drug discovery may be arrived at.

MATERIALS AND METHODS

Study area: This study was carried out at the Laboratory of Molecular Physiology, Division of Gene and Protein Analysis, Central Laboratory, Universitas Padjadjaran, the Laboratory of Animal Biosystem, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran and the Laboratory of Pharmacology, Faculty of Pharmacy, Universitas Padjadjaran, from May, 2018 -January, 2019.

Instruments: Instruments utilized in this work were Eppendorf centrifuge 5425 R, electrophoresis chamber (Mini-PROTEAN Tetra Handcast Systems), protein transfer (Mini Gel Tank Invitrogen Cat. No. A25977), shaker (Biosan MR-1), Western blot scanner (LI-COR C-DiGit Chemiluminescence), microtome (Microm HM 310), microscope (Olympus CX21) and microscope camera (OptiLab Microscope Digital), rotary evaporator (IKA 0010000403 RV 8V rotary evaporator with dry ice condenser), micropipette (Socorex®) and chemical glasswares.

Chemicals and plant materials: Fresh *E. elatior* flowers purchased from Bumi Herbal, Bandung, Indonesia, was taxonomically identified at the Laboratory of Plant Taxonomy, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran (Document No. 198/HB/10/2018). Quercetin hydrate standard 96% purity (Tokyo Chemical Industry Co., Ltd. CAS RN 849061-97-8 Product No. P0042) was purchased from Quartiz Laboratories, Indonesia (a branch of PT Indogen Intertama, Indonesia).

Preparation of *E. elatior* flower extract: The preparation of *E. elatior* flower extract was carried out by following the procedure of Jackie *et al.*[9] with a few modifications using 400 g of dried *E. elatior* flowers.

Phytochemical screening and total phenols determination: The *E. elatior* flower extract was screened for the presence of secondary metabolites such as; alkaloids, phenolic compounds, flavonoids, tannins and saponins using standard procedures of analysis[15]. The phenolic compounds were evaluated equivalent to quercetin using the Folin-Ciocalteu colorimetry method with some modifications[16].

DPPH radical scavenging assay: The antioxidant activity of *E. elatior* flower extract was investigated by using DPPH radical scavenging assay. This assay is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants. The experiment was carried out in triplicates[17]. The IC_{50} was calculated by using GraphPad Prism 8.4.0.

Animals handling and ethical approval: Three months age of healthy male Wistar rats (200-260 g) were purchased from D45 White Rats Experimental Animal Supplier, West Java,

Indonesia. The rats were acclimatized in cages (30×24×10 cm^3, three animals per cage) under standard laboratory conditions (25±2°C, 12 h light/dark cycles) for seven days before treatment. The rats were given access to standard animal diet and water *ad libitum*[18]. All the experimental protocols conducted on rats were performed by following the internationally accepted principles for laboratory animal use and care and were approved by the Universitas Padjadjaran Health Research Ethics Committee (No. 1237/UN6.KEP/EC/ 2018).

Experimental study design and treatment: Animals were divided randomly into 6 groups with 3 animals in each group. The rats have fasted for 24 h: Group 1 was the normal control (water only), group 2 was the negative control (Arabic gum suspension 2%), group 3 was the positive control (quercetin 20 mg kg^{-1} b.wt., suspended in Arabic gum 2%), group 4-6 were treated with *E. elatior* flower extract dose of 500, 1000 and 2000 mg kg^{-1} of b.wt., respectively. About 2 h later, all animal groups, except the normal group were induced with 0.1 mL of acetic acid 1% (v/v) by intraperitoneal injection[19,20]. About 18 h post-induction, the animals were sacrificed. The stomach was taken out for further evaluation[21].

Stomach weight (g kg^{-1} of b.wt.) evaluation: The stomach was collected and weighed for the initial evaluation of its pathological changes due to gastric ulceration-induced by using acetic acid. The stomach weight (g kg^{-1} of b.wt.) of each group was compared to that of the negative control group.

Macroscopic evaluation of gastric mucosal damage: The stomach fundus was exposed and was opened longitudinally to determine the damage scores[21–23].

Microscopic evaluation of gastric mucosal damage: Parts of the rat's stomach were cut and transferred into 10% formalin solution for histological examination. The organ samples were stained using hematoxylin and eosin and were observed under a light microscope (Olympus CX21) coupled with a microscope camera (OptiLab Microscope Digital (40x and 100x magnification) for the presence of inflammatory cells infiltration. The number of inflammatory cells was calculated per 1000x objective lens visibility.

Western blot analysis: The rat's stomach was homogenized and weighed. About 25 g of each gastric protein was extracted using lysis buffer (SDS and Tris-HCl to pH 8.0) and sample buffer (Tris-HCl pH 6.8, glycerol, β-mercaptoethanol, bromophenol blue and distilled water). The samples were heated at 95°C for 5 min, loaded into 10% polyacrylamide gel and run at 150 V for 2 h. The resolved proteins were transferred to a polyvinylidene fluoride membrane using 200 mA for 30 min. The membranes were then incubated with NF-kappaB-p65 polyclonal antibody (1:150, Cat. No. E-AB-32232) (Elabscience Biotechnology Inc., Texas, USA), β-actin antibody HRP conjugate (BA3R) (Cat. MA515739HRP) (Thermo Fisher Scientific Fremont, California, USA) and mouse anti-GAPDH (1:1000) (Cat. #AM4300) (Thermo Fisher Scientific Fremont, California, USA). Eventually, the membranes were washed and incubated with the secondary antibody, chicken anti-rabbit IgG-HRP (1:10000) (Cat. sc-2955) (Santa Cruz Biotechnology Inc., California, USA) and HRP-conjugated chicken anti-mouse (1:10000) (Cat. sc-2954) (Santa Cruz Biotechnology Inc., California, USA). Each band was visualized by enhanced-chemiluminescence substrates (Cat. #926-95000) (Li-Cor Biosciences, Lincoln, USA) and the intensity of the band was measured using Image-J software.

Statistical analysis: Results are expressed as Mean±SD (n = 3). The homogeneity of the data was evaluated using the Levene test. The differences of multiple groups were statistically calculated by one-way ANOVA with a p-value of 0.05, followed by a *post hoc* Bonferroni test (CI 95%). All statistics calculation was performed using IBM SPSS Statistics 20.0 for Windows.

RESULTS

Phytochemical screening and total phenols determination: Phenolics and flavonoids, alkaloids and saponins were positively identified in the *E. elatior* flower extract. The Total

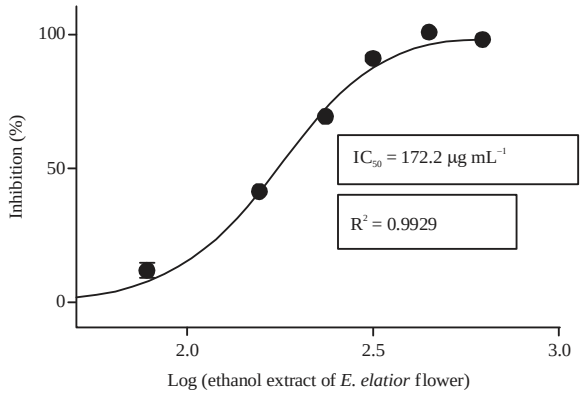

Fig. 1: Log concentration-response curve of the ethanol extract of *E. elatior* flower against inhibition (%) of DPPH radical (calculated by using GraphPad Prism 8.4.0)

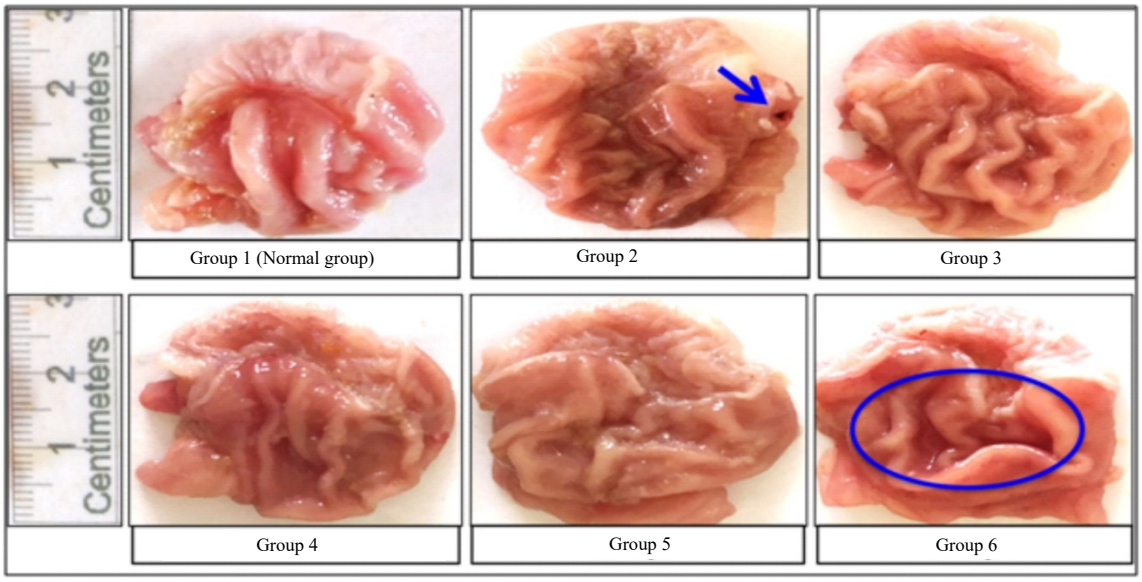

Fig. 2: *Etlingera elatior* flower extract decreases gastric mucosal damage, gastric ulceration is showed by the blue arrow, hyperemia is showed by the blue circle, rats stomach were collected 18 h after acetic acid induction

Table 1: Stomach weight (g kg^{-1} b.wt.±SD; n = 3) of each group

Animal groups	N	Stomach weight (g kg^{-1} b.wt.)±SD
Group 1 (normal group)	3	7±0.5
Group 2 (negative control)	3	5±0.0
Group 3 (positive control)	3	6±1.1
Group 4	3	5±0.5
Group 5	3	6±0.0
Group 6	3	7±0.0

There is no significant difference in stomach weight (g kg^{-1} b.wt.) of all tested groups compared to the negative control

Table 2: Ulceration index

Animal groups	Ulceration index
Group 1 (normal group)	0.00
Group 2 (negative control)	0.67
Group 3 (positive control)	0.00
Group 4	0.33
Group 5	0.50
Group 6	0.83

Phenolic Content (TPC) of the ethanolic extract was 637.80 mg/100 g quercetin equivalent (based on dry extract).

DPPH radical scavenging assay: The IC_{50} of the extract is 172.2 µg mL^{-1} (Fig. 1) that indicates a medium strength of radical scavenging activity.

Stomach weight (g kg^{-1} of b.wt.) evaluation: The stomach weight (g kg^{-1} of b.wt.) of each group is provided in Table 1. The stomach weight of normal control rats (group 1) is 7±0.5 g kg^{-1} b.wt. Acetic acid induction indicated a decrease of the stomach weight (5±0.0 g kg^{-1} of b.wt.) as observed in the negative control rats (group 2). Pre-treatment with *E. elatior* flower extract (dose of 1000 and 2000 mg kg^{-1} of b.wt.) before acetic acid induction could increase the stomach weight almost similar to that of normal control rats. Nonetheless, there is no significant difference in stomach weight (g kg^{-1} of b.wt.) of all tested groups compared to the negative control group.

Table 3: Number of inflammatory cells infiltration in rat gastric tissue observed with objective lens 1000× magnification (mean±SD, n = 3)

Animal groups	N	No. of inflammatory cells infiltration±SD
Group 1 (normal group)	3	3.2±0.58
Group 2 (negative control)	3	7.7±0.58
Group 3 (positive control)	3	5.6±0.58*
Group 4	3	9.1±0.00*
Group 5	3	7.1±1.15
Group 6	3	8.5±0.58*

*Significant difference compared to the negative control ($p<0.05$)

Macroscopic evaluation of gastric mucosal damage: Rats treated with quercetin and lower doses of the extracts (group 3, 4, 5) and did not show ulcerations. However, rats that were given the highest dose of the extract (group 6) showed hyperemia (Fig. 2). Furthermore, rats in group 4 and 5 exhibited lower ulceration index compared to the negative control (group 2). Pre-treatment with lower doses of *E. elatior* flower extract could protect the gastric mucosa from acetic acid-induced damage. The ulceration index is presented in Table 2.

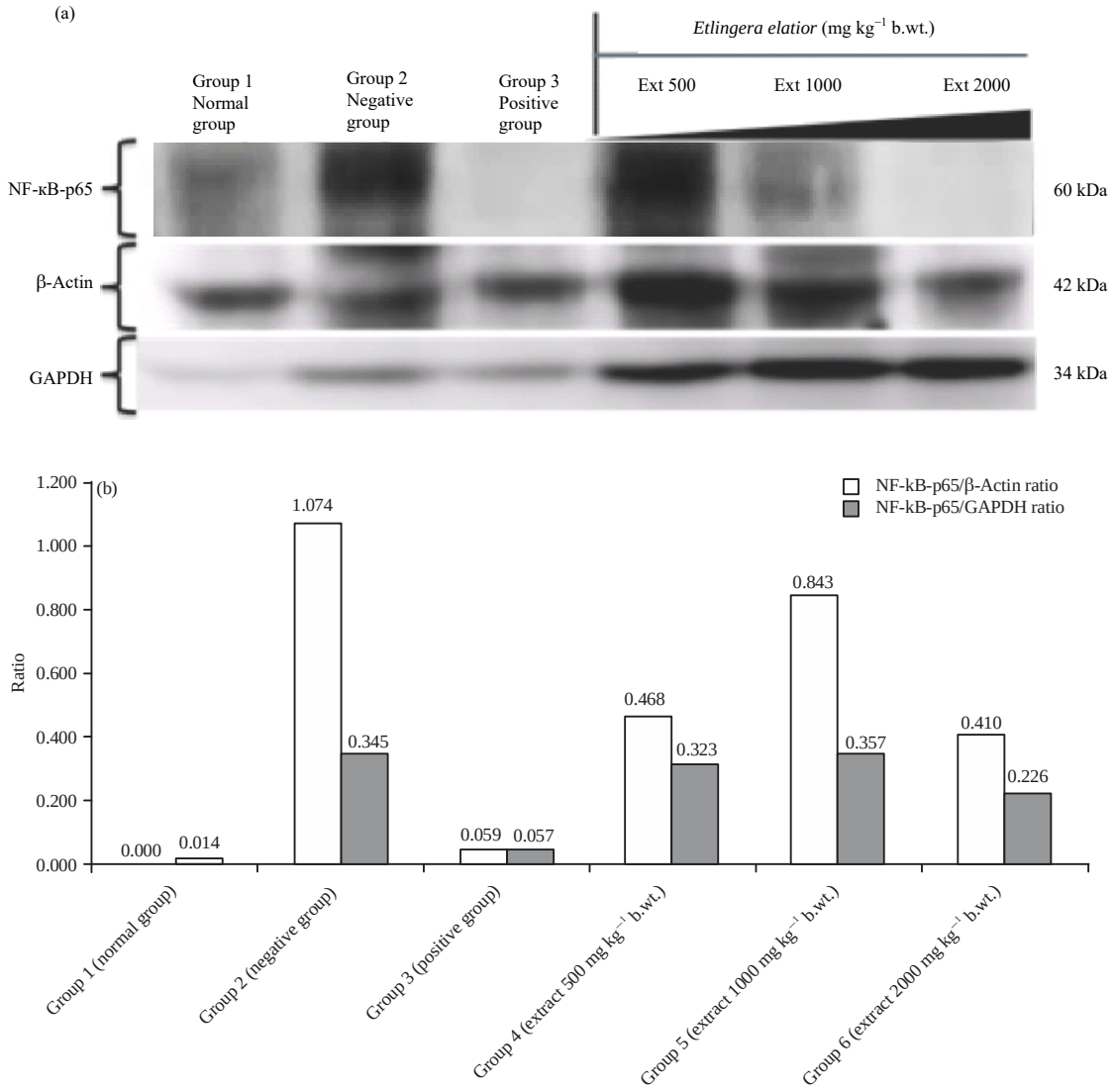

Fig. 3(a-b): Effect of *E. elatior* flower extract on NF-kappaB p65 expression (a) NF-kappaB pathway was explored in the stomach fundus of acetic acid-induced rats using Western blot analysis, β-actin and GAPDH were used as reference control (upper) and (b) Histogram ratio of NF-kappaB-p65/β-actin and NF-kappaB-p65/GAPDH affected by *E. elatior* flower extract (lower)

Microscopic evaluation of gastric mucosal damage: All rats, except in the normal group, experienced mild inflammation as indicated by the presence of 5-10 inflammatory cells. Significant differences compared to the negative control group was shown in group 3, 4 and 5 (Table 3).

***E. elatior* flower extract alters the expression of NF-kappaB-p65 in acetic acid-induced rats:** The NF-kappaB-p65 expression study result is presented in Fig. 3. NF-kappaB p65 is not expressed in the normal group. An elevated ratio of NF-kappaB-p65/β-actin is observed in the negative control group. The *E. elatior* flower extract dose of 1000 and 2000 mg kg^{-1} of b.wt., could down-regulate the expression of NF-kappaB-p65 in the stomach fundus of acetic acid-induced rats.

DISCUSSION

This study revealed that the TPC of the flower of *Etlingera elatior* (Jack) R.M. Smith is 637.80 mg/100 g quercetin equivalent. A comparison with the determination of the TPC in *E. elatior* flower planted in Peninsular Malaysia

indicated that the TPC in *E. elatior* flower harvested from highland and lowland were 3550±304 mg/100 g gallic acid equivalent and 2390±329 mg/100 g gallic acid equivalent, respectively[24]. Moreover, the antioxidant activity of *E. elatior* flower extract indicated a medium strength of DPPH radical scavenging activity (IC_{50} = 172.2 μg mL^{-1}), which might contribute to its ability to improve the macroscopic and microscopic mucosal damage in the gastric ulceration-induced Wistar rats. However, a different result has been reported. The antioxidant activity of *E. elatior* flower planted in Bogor, Indonesia, resulted in IC_{50} = 19.5±0.6 μmoL g^{-1} Trolox equivalent and was categorized as a strong antioxidant[25].

The gastro-protective activity of flavonoids is related to the antioxidant strength. The mechanism of action of flavonoid's antioxidant, especially that of quercetin is mainly due to the presence of the o-dihydroxy ring on ring B (catechol) and strengthened by the existence of double and triple bonds conjugated with 4-oxo function. Moreover, the presence of hydroxyl groups in positions 3, 5 and 7 in its structure contributes to its pharmacology activity[26,27]. The antioxidant activity of quercetin was also studied and it was confirmed that there is a correlation between the antioxidant effect with the anti-ulcerogenic activity of quercetin. Quercetin was reported effective against the oxidative stress-induced gastric damage caused by histamine intraperitoneal injection. This particular flavonoid could increase in the antioxidant enzymatic activity e.g., SOD, CAT and GSH-Px levels in male guinea pigs[28]. Furthermore, a previous study on the formulation of famotidine in combination with solid dispersion of quercetin indicated an improvement in the anti-ulcer activity compared to commercially available tablets[29].

In this study, acetic acid was employed as the gastric ulceration inducer, because the ulcers are often found in humans[19,30]. The acetic acid ulcer model was developed in 1969 and proved that by injecting a single-dose of acetic acid solution into the gastric mucosal layer of rats, mucosal surface damage would occur at 30 min post-injection. Furthermore, an intraluminal injection of the same acid at the rat's fundus mucosa resulted in deep, round ulcers, developed in the area that had been exposed to the acetic acid solution. NF-kappaB is activated by acetic acid induction. Acetic acid induction causes local inflammation, increases inflammatory cytokines, reactive oxygen species and cell damage[31,32]. Acetic acid-induced gastric ulcer also showed an increase in TNF-α and IL-1β which causes activation of the NF-κB pathway in the gastric mucosa and is exacerbated by a decrease of antioxidant activity such as; glutathione, superoxide dismutase (SOD) and catalase activity[32]. Earlier studies indicated that acetic acid activated the NF-kappaB inflammatory signaling pathway due to stimulation by proinflammatory cytokines such as; TNF-α and IL-1β with its receptors[33,34].

This study also presented that the flower of *Etlingera elatior* (Jack) R.M. Smith extract could down-regulate the expression of NF-kappaB-p65. In normal conditions, NF-kappaB is attached to IkappaB in the cytoplasm. When this signaling pathway is activated by a pathogen or proinflammatory cytokines, the complex is degraded and the released-NF-kappaB will translocate to the nucleus. The transcription begins when the NF-kappaB-p65/p50 dimer interacts with a specific DNA in the responsive gene promoter area and eventually regulates the gene transcription[35]. An inflammatory process can be inactivated by inhibiting the translocation of the NF-kappaB-p65 from the cytoplasm to the nucleus[36]. Synthetic drugs that have been proven to work on such mechanisms are celecoxib[37], prednisolone[38] and acetylsalicylic acid[39]. However, these drugs could exacerbate side effects, e.g., immunodeficiency, hormonal disorders and gastrointestinal disorders[40]. Medicinal plants containing quercetin, myricetin and kaempferol have been proven in inhibiting the translocation of the NF-kappaB-p65[41-43].

The limitation of this study is only determining one biomarker, NF-kappaB-p65 for the Western blot analysis. Nevertheless, this study confirmed that there is a correlation between the antioxidant activity of the ethanol extract of *E. elatior* flower with the possible anti-inflammatory mechanism in inhibiting the pathogenesis of gastric ulceration by down-regulating the expression of NF-κB-p65.

CONCLUSION

The ethanol extract of *E. elatior* flower may possess a potential medium DPPH-radical scavenging activity and anti-inflammatory activity in inhibiting the pathogenesis of gastric ulceration by down-regulating the expression of NF-κB-p65 in the stomach fundus of acetic-acid induced gastric ulceration Wistar rats. Thus, contributes in the discovery of plant-based anti-gastric ulceration drugs.

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