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Original Article

Effects of *Etlingera elatior* flower extract on cyclooxygenase-2 expression in the gingival epithelium in a diabetic periodontitis rat model

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الملخص

أهداف البحث: يهدف هذا البحث إلى دراسة تأثير مستخلص زهرة الشعلة الحمراء الإيثانولي بنسبة 70% على تعبير إنزيمات الأكسدة الحلقية-2 في ظهارة اللثة لدى الجرذان المصابة بالتهاب اللثة السكري.

طريقة البحث: تم تحريض الإصابة بمرض السكري والتهاب اللثة في 32 ذكرا من الجرذ النرويجي بوزن 200-300 جرام. تم حقن الستربتوزوتوسين المذاب في 1 مل من محلول السترات داخل الصفاق لتسبب ارتفاع السكر في الدم. بعد ثلاثة أيام من ظهور مرض السكري، تم قياس مستويات السكر في الدم أثناء الصيام لدى الفئران باستخدام مقياس السكر في الدم. تم التأكد من الإصابة بمرض السكري عندما تجاوزت مستويات الجلوكوز في الدم الصائم 200 ملغم / ديسيلتر. بعد تشخيص التهاب اللثة السكري، تم إعطاء حقنة يومية من مستخلص الإيثانول "إي. إليتيور" بنسبة 70% (العدد = 16) والمحلول الملحي (العدد = 16) داخل الصفاق لمدة سبعة أيام. تم إجراء إجراء الكيمياء المناعية للكشف عن تعبير الأكسدة الحلقية-2 في ظهارة اللثة في الأيام 1 و3 و5 و7 بعد الحقن. يتم التعبير عن عدد الخلايا الملونة بشكل إيجابي كنسبة مئوية. شوهد السيتوبلازم ذو اللون البني في ظهارة اللثة يشير إلى تعبير إيجابي لـ الأكسدة الحلقية-2، ويمتد من الطبقة القاعدية إلى الطبقة القرنية.

النتائج: مقارنة بالمحلول الملحي، أدى المستخلص الإيثانولي بنسبة 70% من حقن زهرة الشعلة الحمراء إلى زيادة تعبير الأكسدة الحلقية-2 في الأيام من الأول إلى الخامس. ومع ذلك، في اليوم السابع، أظهرت مجموعة الشعلة الحمراء مستوى منخفضا بشكل كبير من تعبير الأكسدة الحلقية-2 مقارنة بالمجموعة المحقونة بالمحلول الملحي.

الاستنتاجات: في التهاب اللثة الناتج عن مرض السكري، تم العثور على 70٪ من مستخلص الإيثانول زهرة الشعلة الحمراء ليكون عنصرا نشطا مفيدا في علاج تعديل المضيف. يقوم المستخلص الإيثانولي بنسبة 70٪ من زهرة الشعلة الحمراء بتعديل تعبير بشكل إيجابي كنسبة مئوية. شوهد السيتوبلازم ذو اللون البني في ظهارة اللثة يشير إلى تعبير إيجابي لـ الأكسدة الحلقية-2 في ظهارة اللثة لدى الجرذان المصابة بالتهاب اللثة السكري.

الكلمات المفتاحية: إنزيمات الأكسدة الحلقية -2؛ مرض السكري؛ أمراض اللثة؛ الكيمياء المناعية؛ زهرة الشعلة الحمراء

Abstract

Objectives: This research was aimed at investigating the effects of 70% ethanolic *Etlingera elatior* flower extract on cyclooxygenase-2 (COX-2) expression in the gingival epithelium in rats with diabetic periodontitis.

Methods: Diabetes and periodontitis were induced in 32 male *Rattus norvegicus* individuals weighing 200–300 g each. Streptozotocin dissolved in 1 mL citrate buffer was injected intraperitoneally to elicit hyperglycemia. Three days after diabetes induction, the rats' fasting blood glucose levels were measured with a GCU EasyTouch® glucometer. Diabetes was confirmed by fasting blood glucose levels exceeding 200 mg/dL. After diagnosis of diabetic periodontitis, a daily injection of 70% ethanolic *E. elatior* extract (n = 16) and saline (n = 16) was intraperitoneally administered for 7 days. Immunohistochemistry was used to detect COX-2 expression in the gingival epithelium on days 1, 3, 5, and 7 after injection, and the number of positively colored cells was expressed as a percentage. Brownish cytoplasm in the gingival epithelium was considered to indicate positive COX-2 expression, which extended from the basal layer to the corneum. The percentage of immunopositive cells was

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analyzed with two-way ANOVA followed by post-hoc LSD analysis at a 95% significance level.

Results: Injection of 70% ethanolic extract of *E. elatior* flower, compared with saline, resulted in greater COX-2 expression on days 1–5. On day 7, however, the *E. elatior* group exhibited substantially lower COX-2 expression than the saline group ($p < 0.05$).

Conclusions: In diabetic periodontitis, 70% ethanolic *E. elatior* extract was found to be a useful active component for host modulation therapy. The 70% ethanolic extract of *E. elatior* flower modulated COX-2 expression in the gingival epithelium in rats with diabetic periodontitis.

Keywords: Cyclooxygenase-2; Diabetes mellitus; *E. elatior*; Immunohistochemistry; Periodontal disease

Introduction

Diabetes mellitus (DM) encompasses a group of metabolic disorders that significantly contribute to morbidity and premature mortality worldwide, and thus is a major health issue. Projections suggest that 366 million people will have DM by the year 2030, primarily in low- and middle-income nations. On a global scale, DM is an epidemic disease. The condition is distinguished by persistently high blood glucose levels. Moreover, in DM, deficits in insulin secretion, insulin activity, or both lead to disruptions in the metabolism of carbohydrates, fats, and proteins.[1–3] Ninety percent of all people with diabetes have type 2 diabetes (T2DM), the most prevalent form of diabetes in the general population.[4]

Periodontitis is the sixth most frequent complication associated with T2DM. Tooth loss may result from the irreversible damage caused by periodontitis.[4] Additionally, periodontal tissue injury is notably more severe in individuals with rather than without T2DM.[5] Periodontitis continues to affect patients with diabetes who maintain excellent oral hygiene.[6] As with all inflammatory processes, including periodontal inflammation, elevated blood glucose levels mediate the reciprocal relationship between diabetes and periodontitis.[7] The inflammation resulting from periodontal infection has the potential to both initiate and worsen hyperglycemia. In contrast, hyperglycemic conditions resulting from T2DM induce disruption of the inflammatory response, thereby increasing the likelihood of periodontitis onset and progression.[4]

Conventional therapy for diabetic periodontitis is ineffective and causes adverse effects.[8] Treatment for periodontitis in individuals with diabetes is often initiated after blood glucose levels have been effectively managed. Medication adherence for a predetermined duration contributes to the regulation of blood glucose levels. The ideal treatment for periodontitis is frequently postponed, and periodontitis may even worsen during blood glucose management. Under persistent inflammation, the management of blood glucose levels becomes challenging. In periodontitis accompanied by T2DM, inflammatory control is frequently suboptimal, because of diminished function of inflammatory cells, including phagocytosis of periodontal pathogens by neutrophils. Consequently, inflammation endures and is further exacerbated by the inhibitory effects of inflammatory mediators released previous inflammatory cells on insulin receptors. Cyclooxygenase-2 (COX-2) is a primary mediator of cytokine excretion during the inflammatory process and an important marker of inflammation.[9]

With the advancement of knowledge regarding the reciprocal relationship between DM and periodontitis, the therapeutic strategy for patients with DM and periodontitis has evolved to include host modulation therapy, which specifically targets inflammation control, in addition to the elimination of local factors. Researchers are developing treatments with natural ingredients to decrease the risk of long-term adverse effects. One natural material under development is *Etlingera elatior*, an extensively cultivated plant indigenous to Indonesia. The flavonoids in *E. elatior* possess anti-inflammatory and potentially hypoglycemic properties. A toxicity study conducted by Juwita et al.[10] has evaluated the pharmacological activity and ingredients of *E. elatior* extract, and suggested its biological safety. However, few studies have examined the effects of *E. elatior* on inflammatory mediators, specifically COX-2, in the context of diabetic periodontitis. The purpose of this research was to determine whether ethanolic *E. elatior* extract, administered as a host modulation therapy, might affect the expression of COX-2 in diabetic periodontitis. The results of this research may help provide a scientific basis for the formulation of *E. elatior*, a naturally occurring substance that may be used to control the inflammation associated with diabetic periodontitis.

Materials and Methods

The research was conducted in a three-step process, consisting of preparation of ethanolic *E. elatior* extract, induction of diabetic periodontitis, and administration of *E. elatior* extract.

Plant species determination

Raw *E. elatior* flowers were collected from Ngaglik, Sleman, Yogyakarta, Indonesia, because of availability and ease of access. The analysis was performed at the Laboratory of Plant Systematics, Indonesia. *E. elatior* flowers were identified as *E. elatior* during species determination.

Preparation of ethanolic E. elatior *extract*

The extract was obtained through a maceration process, in which 2000 g *E. elatior* flower was cleaned, dried in an oven at 45 °C for 48 h, and ground into fine powder. The fine powder was mixed with 70% ethanol and stirred for 24 h until dissolved. A previous investigation indicated that 70% ethanol extract, compared with pure ethanol extract,

contains a higher number of bioactive flavonoid molecules, because of its higher polarity.[11] The solvent was replenished every 24 h at room temperature. This procedure was repeated twice, and the final product was concentrated with a rotary evaporator at 40 °C, thus yielding 22.60 g of viscous extract.

Phytochemical screening of flavonoids

Thin layer chromatography (TLC) was used to screen for flavonoids in the ethanolic *E. elatior* extract. The stationary phase of the TLC plate/silica gel, measuring 8 cm long and 2 cm wide, was cleaned with methanol and activated in an oven at 100 °C for 10 min. The extract samples were spotted in the stationary phase. Ammonia vapor stains appeared in the mobile phase, which comprised a 1:4:5 ratio of glacial acetate to butanol to water. Brownish-yellow stains after steaming with ammonia under blue light at UV 366 nm indicated the presence of flavonoids.

Induction of diabetic periodontitis

The *in vivo* experimental study included 32 male *Rattus norvegicus* individuals; each weighed 200−300 g, was 2−3 months of age, and developed diabetic periodontitis. *Ad libitum* feeding was provided for rats. Rats were sedated with ketamine (80 mg/kg BW) and xylazine (10 mg/kg BW) before diabetic periodontitis induction. Streptozotocin dissolved in 1 mL citrate buffer was administered intraperitoneally at 40 mg/kg BW to induce hyperglycemia. Three days after diabetic induction, the fasting blood glucose levels of the rats were evaluated with a GCU EasyTouch® glucometer. DM was confirmed when the fasting blood glucose levels exceeded 200 mg/dL.

Concurrently with DM induction, periodontitis was induced by winding of a #3/0 silk ligature around the mandibular incisor and leaving the ligature in place for 7 days. Ligature-induced periodontitis in rats is characterized by elevated gingival inflammation; bleeding on probing; and tooth mobility,[12] a clinical signs of bone resorption.[13]

Administration of E. elatior *extract*

Once per day for 7 days, *E. elatior* extract (100 mg/kg BW) was administered via peritoneal injection. A prior study has indicated that this dose decreases diabetic rats' blood glucose levels.[14] For 7 days, 0.2 mL *E. elatior* extract was administered intraperitoneally once per day through insertion of a 1 mL needle cranially to the abdomen, back of the umbilical cord, and lateral areas of the body or midline. This simple process enables medications to be supplied and absorbed quickly.[15]

Immunohistochemistry procedure and analysis

After euthanasia on days 1, 3, 5, or 7, collected tissues were paraffinized and decalcified. COX-2 expression in the gingival epithelium was determined through immunohistochemistry (Elabscience IHC reagent, 2-step plus Poly-HRP Anti-Mouse/Rabbit IgG Detection System, USA). Brown color indicated COX-2 positivity. The percentages of COX-2 immunopositive cells were examined as described in a previous study,[16] by two independent observers using an Olympus Microscope CX23 Tokyo Japan, at ×400 magnification, with five fields of view. To evaluate interobserver reliability, we used the Bland−Altman test. Data analysis was performed with two-way ANOVA followed by a post hoc LSD test ($p < 0.05$).

Results

TLC confirmed the flavonoid content of ethanolic *E. elatior* flower extract. Brownish-yellow stains indicated the presence of flavonoids (Figure 1).

Three days after diabetes induction, the rats' mean fasting blood glucose levels were 301 mg/dL, thus demonstrating the presence of diabetes. All rats also displayed clinical manifestations of diabetes, including polydipsia, polyuria, polyphagia, and malaise. After 7 days, all rats demonstrated clinical symptoms consistent with periodontitis, such as gingival inflammation, bleeding upon probing, and tooth mobility.

COX-2 positivity was observed according to the presence of brown color (yellow arrow) in the cytoplasm of the gingival epithelium, from the basal layer to the stratum corneum (Figure 2). The percentage of positively colored cells was counted (Table 1). The Shapiro−Wilk test indicated that the data were normally distributed ($p > 0.05$), and the Levene test indicated that the data were homogeneous ($p > 0.05$).

Figure 1: Thin layer chromatography analysis of *E. elatior* flavonoids.

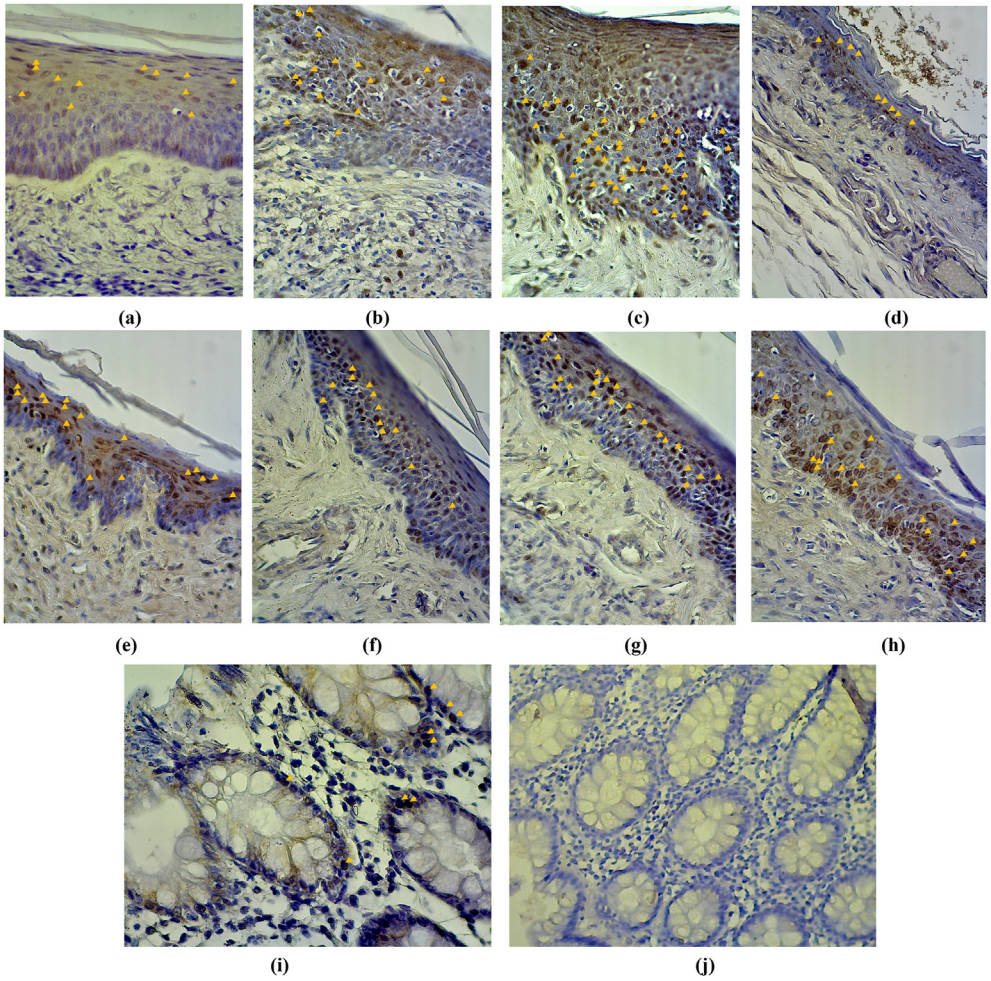

Figure 2: COX-2 immunohistochemical staining (×400 magnification) in the *E. elatior* group at days 1 (a), 3 (b), 5 (c), and 7 (d); in the saline group at days 1 (e), 3 (f), 5 (g), and 7 (h); and in tonsil tissue positive control (i) and tonsil tissue negative control (j). Yellow arrows indicate immunopositive cells.

Table 1: Percentage of COX-2 immunopositive cells in the studied groups.

Day	*E. elatior*		Saline		*p*-value
	Mean	SD	Mean	SD	
1	38.3993	3.3146	34.4898	2.2585	0.000*
3	43.8297	1.6631	35.6164	2.8399	
5	36.8727	0.7137	33.4286	1.0346	
7	31.8780	0.7584	36.6378	0.7562	

SD, standard deviation.
Tested with; two-way ANOVA.
*$P < 0.05$, significant difference between groups.

The percentage of COX-2 immunopositive cells in the *E. elatior* group on days 1, 3, and 5 was higher than that in the saline group ($p < 0.05$; Table 1). However, COX-2 expression in the *E. elatior* group decreased on day 7 but continued to increase in the saline group ($p < 0.05$). The post hoc LSD analysis results are shown in Table 2.

The Bland–Altman test indicated mean and standard deviation values of 1.66 and 4.89, respectively. The upper range was 11.25, and the lower range was −7.92. Based on the scatter plot analysis, it was observed that certain plots had values that were both higher and lower; consequently, a regression test was performed and revealed a coefficient B

Table 2: Post hoc LSD analysis of COX-2 immunopositive cells in the studied groups.

	E1	S1	E3	S3	K5	S5	K7	S7
E1		0.008*	0.001*	0.052	0.273	0.001*	0.000*	0.208
S1			0.000*	0.416	0.093	0.443	0.067	0.128
E3				0.000*	0.000*	0.000*	0.000*	0.000*
S3					0.365	0.121	0.011*	0.460
E5						0.018*	0.001*	0.864
S5							0.266	0.027*
E7								0.002*
S7								

E1: *E. elatior* Day 1
E3: *E. elatior* Day 3
E5: *E. elatior* Day 5
E7: *E. elatior* Day 7
S1: Saline Day 1
S3: Saline Day 3
S5: Saline Day 5
S7: Saline Day 7
(*): significant if p value <0.05

value of nearly 0 with p = 0.885 ($p > 0.05$). Therefore, we concluded that both independent observers had good reliability.

Discussion

Diabetic periodontitis, a chronic inflammatory condition affecting the gingiva in diabetes and supporting dental structures, is caused by a complex combination of variables including hyperglycemia, microbial factors, an impaired immune response, and excessive inflammatory mediators. The rats in the present study showed clinical outcomes of diabetes, including polydipsia, polyuria, polyphagia, and malaise, along with elevated fasting blood glucose (>200 mg/dL).

Despite the lack of data on fasting blood glucose after extract administration, we suspect that this extract might have a hypoglycemic effect, because an earlier study reported that *E. elatior* administration at 100 mg/kg BW decreases blood glucose levels in diabetic rats.[17] This antidiabetic activity may be attributable to the anthocyanin antioxidants cyanidin-3-O-glycosides, the active compound. Jackie et al.[17] have suggested that *E. elatior* may potentially be used as a natural source of antioxidants for the prevention or treatment of DM[18] and diabetic nephropathy.[19] Furthermore, *E. elatior* flower demonstrated a dose-dependent antihyperglycemic effect, thus substantially decreasing the elevated total cholesterol, triglyceride, and low-density lipoprotein levels. Alkaline phosphatase, aspartate aminotransferase, and serum creatinine all proved significantly reduced in comparison to controlled DM.[18]

Periodontitis, a common oral complication in diabetes, has a prevalence of 67.8%.[20] In the present study, we induced periodontitis through ligature. The application of ligatures elicits rapid onset inflammation and resorption of alveolar bone. Our study confirmed the presence of periodontitis, on the basis of clinical signs such as increased gingival inflammation; bleeding on probing; and tooth mobility,[12] a clinical sign of bone resorption.[13]

Diabetic periodontitis is frequently characterized by inflammation, a process notably facilitated by COX-2, through modulation of the recruitment of additional inflammatory mediators including IL-1, IL-6, and TNF-α.[21–23] High blood glucose levels increase COX-2 expression, thereby contributing to diabetic periodontal disease progression[24,25] through the activation of inflammatory pathways such NF-κB and MAPKs.[26] Elevated COX-2 levels promote prostaglandin synthesis, including prostaglandin E2 (PGE2), which contributes to inflammation and tissue damage in periodontal tissues.[24,27] PGE2 increases vascular permeability and bone resorption.[28] Therefore, COX-2 is frequently used as a biomarker of inflammatory conditions in DM.

In DM, inflammation control requires timely, consistent, and comprehensive attention. COX 1 and COX 2, the primary mediators of inflammatory processes in the body, induce the synthesis of prostaglandins via Tyr385 and/or Ser530 in COX-2, and Arg120 and/or Tyr355 in COX-1. The constitutive cyclooxygenase-1 and the inducible COX-2 are well distinguished. Nonsteroidal anti-inflammatory drugs are targeted inhibitors of both enzymes and are the most frequently prescribed anti-inflammatory agents. Nevertheless, the anti-inflammatory medications that are currently in use have been linked to a variety of adverse effects that could potentially lead to a decrease in production or even their withdrawal from the market.[29] Scientific evidence has highlighted the links between periodontal diseases and diabetes. Diabetes, which is defined by elevated glycated hemoglobin (HbA1c) levels, has been incorporated as a descriptive factor in the categorization of periodontal diseases.[30] Thus, efficacious treatments must be developed for patients with periodontitis and DM. Despite the demonstrated efficacy of scaling and root planing, as components of periodontal treatment, in decreasing HbA1c levels, novel approaches have emerged, such as modulating the inflammatory host response.[31] An increasing number of herbal constituents are being used to modulate the host's inflammatory response to counteract adverse effects. Derivatives of naturally occurring ω-3 fatty acids generated by COX-2 have been reported to be anti-inflammatory mediators.[28]

E. elatior is a naturally occurring substance exhibiting anti-inflammatory properties.[10] Immune system regulation substantially affects inflammation control in diabetic periodontitis. The current findings demonstrated the immunomodulatory ability of *E. elatior*, on the basis of an increase in COX-2 expression from days 1−5, followed by a decline in expression on day 7. We hypothesized that the inflammatory response was accelerated by the upregulation of COX-2 expression from day 1 to day 5. The increase in

COX-2 expression might have been caused by the body reacting to exogenous substances or the dose of *E. elatior* having been sufficient to inhibit COX-2 expression during the first 5 days. On day 7, *E. elatior* inhibited COX-2 expression more effectively than saline. Prior investigations have yielded similar findings, in which until day 7, *E. elatior* administration improved immunomodulatory effects protecting against the development of systemic and local inflammatory conditions.[10,21]

A substantial decrease in COX-2 expression is expected to accelerate tissue regeneration.[21–23] It also serves as evidence of its effectiveness in preventing the progression of inflammation. Because COX-2 is activated predominantly in response to inflammatory stimuli, selective inhibition of COX-2 might potentially mitigate inflammation without affecting the physiological functions of COX-1-derived PGsc.[32,33] However, the present investigation did not provide evidence supporting this hypothesis, because of the lack of COX-1 expression data. Additional research is necessary to observe the interaction between COX-1 and COX-2, to comprehensively elucidate the anti-inflammatory mechanism of *E. elatior* flower extract.

E. elatior was found to inhibit the expression of COX-2 in the gingival epithelium, thus demonstrating anti-inflammatory characteristics in diabetic periodontitis. This phenomenon might be associated with secondary metabolite compounds, such as quercetin, phenols, flavonoids, glycosides, saponins, tannins, steroids, and terpenoids, within the flowers of *E. elatior*.[34,35] Flavonoids present in *E. elatior* exhibit antihyperglycemic activity via the inhibition of α-glucosidase and α-amylase enzymes, in addition to anti-inflammatory properties. Inhibition of these enzymes restricts carbohydrate absorption and decreases postprandial sugar absorption, thus aiding in the regulation of hyperglycemia in diabetes and decreasing blood glucose levels.[10] Flavonoids are signaling molecules that interact with specific proteins central and subsequently disrupt intracellular signaling cascades. *E. elatior* inhibits the activity of enzymes involved in regulating the inflammatory response. These enzymes have roles in prostanoid biosynthesis, histamine release, phosphodiesterase, protein phosphorylation, and transcriptional activation. Although the present investigation only verified the flavonoid content but did not examine flavonoid isolates, *E. elatior* may be likely to have similar anti-inflammatory properties to those of other members of the same family, such as red ginger (*Zingiber officinale* var. Rubrum).[10] Beyond its antioxidant and anti-inflammatory properties, the ethanol extract of the *E. elatior* fruit exhibits anti-hypertensive effects. The extract contains natural anti-inflammatory and antioxidant compounds that contribute to its nephroprotective properties.[18,19] Moreover, the anti-inflammatory properties of *E. elatior* have been demonstrated in animal models of sepsis.[36] The extract's impressive antioxidant activity and high concentrations of total phenolic and flavonoid compounds have been hypothesized to contribute to its antidiabetic properties.[37] In summary, by regulating COX-2 expression, *E. elatior* flower has the potential to serve as a natural source of anti-inflammatory and anti-hyperglycemic agents for the prevention or treatment of diabetic periodontitis.

Conclusion

The current study indicated that the 70% ethanolic extract of *E. elatior* flowers regulates COX expression −2 in the gingival epithelium in rats with diabetic periodontitis. Despite the study's limitations, the current findings may serve as a starting point for the development of 70% ethanolic *E. elatior* extract as an active component of host modulation therapy in diabetic periodontitis. Additional research regarding the dosage and formulation is required.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

The Ethics Committee of FKG-RSGM UGM, Indonesia approved this investigation under reference numbers 172/KE/FKG-UGM/EC/2022 and 141/UN1/KEP/FKG-RSGM/EC/2023.

Authors contributions

AS, RS, and AAA conceived and designed the study, conducted research, provided research materials, and collected and organized data. AS and RS analyzed and interpreted data. AS, RS, and AAA wrote the initial and final drafts of the article. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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