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Research Article

Antidiabetic and TNF-α Lowering Effect of *Tagetes erecta* Extract in Alloxan-Induced Diabetic Rats

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Abstract

Diabetes mellitus is a chronic metabolic disorder characterized by impaired insulin production or action, often leading to pancreatic β-cell dysfunction and apoptosis. Pro-inflammatory cytokines, notably Tumor Necrosis Factor-alpha (TNF-α), play a critical role in the pathogenesis of type 2 diabetes (T2D). While *Tagetes erecta* (marigold) has demonstrated potential in lowering blood glucose in hyperglycemic conditions, its anti-inflammatory effects in diabetic models remain underexplored. This study aimed to evaluate the antidiabetic and TNF-α lowering effects of *T. erecta* extract in alloxan-induced diabetic rats. Twenty-five male Wistar rats were divided into a normal control group (n=5) and a diabetic group (n=20) induced by alloxan (blood glucose ≥126 mg/dL). Diabetic rats were then randomized into four treatment subgroups (n=5 each): untreated diabetic control, and diabetic groups treated with *T. erecta* extract at doses of 25 mg/kg BW, 50 mg/kg BW, or 75 mg/kg BW (administered intraperitoneally). Statistical analysis revealed that *T. erecta* extract significantly reduced blood glucose levels in alloxan-induced diabetic rats ($p < 0.05$). Furthermore, the highest dose of *T. erecta* extract (75 mg/kg BW) effectively attenuated elevated TNF-α levels, demonstrating a significant anti-inflammatory effect. In conclusion, this study provides compelling evidence that *T. erecta* extract exhibits both antidiabetic and anti-inflammatory properties by significantly lowering blood glucose and TNF-α levels in alloxan-induced diabetic rats, particularly at the 75 mg/kg BW dose.

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INTRODUCTION

Diabetes mellitus (DM), a prevalent chronic metabolic disorder, is characterized by elevated blood glucose levels exceeding physiological norms[1]. While Type 1 (T1DM) and Type 2 (T2DM) diabetes mellitus exhibit distinct underlying mechanisms, both converge on a fundamental defect: the dysfunction or apoptotic demise of insulin-producing pancreatic β-cells. This β-cell demise is frequently exacerbated by inflammatory cytokines, which directly contribute to cellular damage and death[2]. The global burden of DM is substantial and escalating. In 2014, 8.5% of adults aged 18 years and older were diagnosed with diabetes. By 2019, the disease was directly responsible for 1.5 million deaths worldwide, with a concerning 48% occurring prematurely before the age of 70. Between 2000 and 2019, a marked increase in diabetes cases was observed across all age groups, with lower-middle-income countries experiencing a particularly high diabetes-related mortality rate of 13%[3]. Type 2 diabetes mellitus accounts for over 90-95% of all diabetes cases and is characterized by a complex interplay of altered lipid metabolism, insulin resistance, and progressive pancreatic β-cell dysfunction[4]. Given this inherent complexity, preclinical

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animal models that accurately recapitulate the multifaceted pathogenesis of the disease are crucial for effective diabetes research[4,5]. Furthermore, inducing inflammation in these animal models is paramount for discovering novel therapeutic and curative strategies[6]. Alloxan, a commonly employed diabetogenic agent, is frequently utilized to evaluate the antidiabetic potential of both pure compounds and plant extracts in diabetes studies[7,8].

Among the key pro-inflammatory cytokines implicated in the pathogenesis of T2DM, Tumor Necrosis Factor-alpha (TNF-α) plays a pivotal role[9,10]. TNF-α is primarily secreted by various immune cells, including macrophages, $CD4^+$ lymphocytes, natural killer (NK) cells, neutrophils, eosinophils, and mast cells, contributing significantly to systemic inflammation and acting as an acute-phase reactant in DM pathogenesis[11]. Research indicates that TNF-α can downregulate the expression of insulin-regulated glucose transporter type 4 (GLUT4) in adipocytes, skeletal muscle, and cardiac tissue[9]. Moreover, TNF-α initiates tissue inflammation through the generation of reactive oxygen species and the induction of specific transcriptional pathways[12]. An imbalance characterized by excessive production of pro-inflammatory cytokines and diminished anti-inflammatory cytokine synthesis prolongs the inflammatory process, leading to uncontrolled tissue damage[13]. Consequently, preventing inflammation by strategically blocking the inflammatory response represents one of the most effective therapeutic avenues for curtailing the development of insulin resistance and mitigating T2DM pathogenesis. Anti-TNF-α treatment strategies, achieved through neutralization or blockade of TNF-α expression, have shown promise in managing insulin resistance and T2DM[14]. The critical role of TNF-α in diabetes pathophysiology continues to be an active area of contemporary research[9].

Current pharmacological treatments for DM predominantly rely on synthetic chemical drugs, which, despite their efficacy, are often associated with numerous adverse effects such as gastrointestinal disturbances, headaches, hematological abnormalities, thrombocytopenia, and agranulocytosis[15,16]. This has prompted a growing interest in herbal medicines as a safer and more accessible alternative, particularly given that the secondary metabolites found in medicinal plants can often be synthetically reproduced[17]. The World Health Organization (WHO) estimates that approximately 80% of the global population utilizes herbal medicines for their primary healthcare needs[18]. Among the diverse array of natural sources, edible flowers, a category of ornamental plants whose flowers and leaves can be consumed[19], have emerged as valuable sources of bioactive compounds. For instance, flower extracts have been found to contain lutein, a biologically beneficial compound with potential as a nutritional supplement[17].

Tagetes erecta, commonly known as African marigold or "Gemitir" in some regions[20], owes the vibrant yellow hue of its flowers to the presence of two primary pigment classes: flavonoids and carotenoids[21]. *Tagetes erecta* flowers are particularly rich in carotenoids, flavonoids, and other phenolic compounds, establishing them as a significant natural source of xanthophylls, including zeaxanthin, lutein, and lutein esters[22]. Recent studies have begun to highlight the antidiabetic benefits of *T. erecta*[18], with lutein derived from its extract demonstrating the potential to reduce blood sugar levels in hyperglycemic mouse models[23]. Despite this promise, research specifically on the anti-inflammatory effects of *T. erecta* extract in Indonesia remains limited. Previous Indonesian studies have predominantly focused on its applications in agricultural development[24], yield and characteristic assessments[25], antioxidant properties[26,27], and hypolipidemic effects[28]. A recent study by Park *et al*.[29] further underscored the therapeutic potential, showcasing *T. erecta* gold nanoparticles synthesized using marigold extract as a promising strategy to mitigate retinal pigment epithelial (RPE) damage induced by high glucose-mediated oxidative stress in diabetic retinopathy. Therefore, the objective of this study was to elucidate the anti-inflammatory effect of *T. erecta* flowers by evaluating the expression of TNF-α as a key inflammatory marker using immunohistochemical methods in an animal model of diabetes.

MATERIALS AND METHODS

Materials

This true experimental study utilized 25 male Wistar strain *Rattus norvegicus*. All animal procedures were conducted in strict accordance with ethical guidelines and were approved by the Health Research Ethical Clearance Commission of Faculty of Dental Medicine, Universitas Airlangga (Approval Number: 959/HRECC.FODM/VIII/2023). The instrumentation and reagents employed in this research included a digital scale, standardized rat cages, a gastroesophageal probe for oral gavage, blood lancets, alcohol swabs, and an Easytouch glucometer for rapid blood glucose assessment. Sample containers were

used for biological specimen collection. Chemical reagents comprised ethanol p.a., 0.9% NaCl solution, sodium carboxymethyl cellulose (Na-CMC), alloxan monohydrate, and 10% neutral buffer formalin (NBF) for tissue preservation. Additionally, TNF-α antibody was used for specific analyses. The plant material, *T. erecta*, were meticulously collected from Buleleng, Denpasar. Botanical identification was formally confirmed at the Laboratorium Herbal Materia Medica Batu, and is documented under registered number 000.9.3/2375/102.20/2023.

Methods

Extraction

Tagetes erecta flowers were dried in an oven at 50 ± 5°C for 9 hours. The dried flowers were then individually ground into a fine powder using a grinder and stored in an airtight container at room temperature for subsequent analyses[25]. For extraction, 400 g of the *T. erecta* powder were macerated in ethanol p.a. at a ratio of 1:5 (w/v) for 24 hours. Following filtration, the resulting extracts were concentrated to dryness using a vertical coiled evaporator condenser at 48−50°C for 2 hours[16].

Phytochemical screening

Qualitative phytochemical screening was conducted to identify the presence of key secondary metabolites within the *T. erecta* extract. This involved a series of established colorimetric and precipitation reactions designed to detect specific chemical groups. Briefly, aliquots of the extract were treated with various reagents, and the resulting color changes or precipitate formations were observed. These characteristic reactions allowed for the preliminary identification of different classes of secondary metabolites, such as alkaloids, flavonoids, terpenoids, tannins, anthraquinones, and saponins, which are known to contribute to the biological activities of plant extracts. The specific methodologies employed for each class of compound were adapted from well-established protocols in phytochemical analysis[30].

Alloxan-induced diabetic models and T. erecta treatment

Twenty-five healthy Wistar rats were randomly allocated into two primary groups. A normal control group consisted of five rats, while the remaining twenty rats formed the diabetic group. To establish baseline glucose levels, random blood glucose was measured in all rats on Day 0. Diabetes was then induced in the twenty rats of the designated diabetic group via a single intraperitoneal injection of alloxan (150 mg/kg BW), freshly dissolved in 0.9% physiological NaCl solution. To confirm the onset of diabetes, blood glucose levels were re-measured 72 hours post-induction (Day 3). Rats with blood glucose levels exceeding or equal to 126 mg/dL were considered diabetic and subsequently included in the treatment phase of the study. The confirmed diabetic rats were then further subdivided into four experimental groups, each consisting of five animals (n=5): a diabetic control group, and three treatment groups receiving *T. erecta* extract at doses of 25 mg/kg BW, 50 mg/kg BW, and 75 mg/kg BW, respectively. These extracts were administered intraperitoneally[31].

Blood glucose test

Blood glucose levels were meticulously monitored throughout the study to assess the progression of diabetes and the efficacy of treatments. Baseline measurements were taken the day prior to treatment initiation. Subsequent measurements were recorded on Day 3 (post-alloxan induction), Day 7, Day 14, Day 21, and Day 28. All blood glucose measurements were performed using an EasyTouch® GCU glucometer (Type ET-301, Bioptik Technology, Taiwan), with readings concurrently confirmed using glucose indicator sticks (Bioptik Technology, Taiwan). The EasyTouch system was chosen for its demonstrated accuracy and precision across a broad spectrum of glucose concentrations[32], ensuring reliable data for analysis.

Preparation and immunohistochemical staining of pancreas organ

On the 28th day of the study, rat pancreases were carefully excised via abdominal and thoracic cavity surgery. Each isolated organ underwent a meticulous hydration process, followed by thorough cleaning with xylene solution to remove residual lipids. The cleaned tissue was then infiltrated with paraffin wax, and subsequently embedded into a paraffin block. Sections of the paraffin-embedded pancreas were cut to a thickness of 5 µm using a microtome. For immunohistochemical analysis, the resulting tissue sections were carefully mounted onto glass slides. Prior to antibody staining, the sections were deparaffinized using xylene solution and rehydrated through a graded series of ethanol baths. Following rehydration, the pancreatic tissue was thoroughly washed with phosphate-buffered saline (PBS). Subsequently, the sections were incubated with primary anti-TNF-α antibodies. After incubation, unbound primary antibodies were removed by washing with PBS.

The sections were then treated with appropriate secondary antibodies conjugated with peroxidase enzymes, followed by another series of washes with PBS. To visualize the immunoreaction, the pancreatic tissue was immersed in 3,3'-diaminobenzidine (DAB) chromogen solution. Counterstaining was performed using hematoxylin, and the slides were then washed under running water. Finally, the sections underwent dehydration, were cleared, mounted with a coverslip, and observed under a light microscope for analysis[33].

Data analysis

All quantitative data are presented as the average ± standard error (SE). To assess statistically significant differences between experimental groups, a One-Way Analysis of Variance (ANOVA) was performed. In cases where the assumption of homogeneity of variances was violated, Tamhane's T2 post-hoc multiple comparison test was employed. A p-value of less than 0.05 was set as the threshold for statistical significance.

RESULTS AND DISCUSSION

The phytochemical screening of *T. erecta* extract was conducted to confirm the presence of key secondary metabolite compounds. Consistent with prior research indicating *T. erecta* as a rich source of bioactive compounds[34], our analysis (**Table I**) revealed the presence of a diverse array of phytochemicals, including alkaloids, flavonoids, terpenoids, tannins, anthraquinones, and saponins. These findings corroborate the traditional uses and previously reported pharmacological activities of this plant.

Table I. Phytochemical screening.

Phytochemical tests	Reagents	Results
Alkaloids	Mayer's reagent	+
Flavonoids	NaOH (Alkaline reagent test)	+
Terpenoids	Acetic anhydride & H_2SO_4 (Salkowski test)	+
Tannins	1% $FeCl_3$ (Ferric chloride test)	+
Anthraquinones	Ammonia (Bornträger's test)	+
Saponins	Water (Froth test)	+

Alloxan monohydrate was employed as a diabetogenic agent in this study due to its well-established ability to selectively induce pancreatic β-cell dysfunction, leading to reduced insulin production and subsequent hyperglycemia[35]. As anticipated, the alloxan-induced group exhibited a significant increase in blood glucose levels, exceeding 126 mg/dL, when compared to the non-alloxan control group (**Table II**). This confirms the successful establishment of the diabetic model, providing a suitable platform for evaluating the anti-diabetic potential of *T. erecta* extract.

Table II. Blood glucose levels in alloxan-induced diabetic rats.

Groups	Blood glucose average ± SE		
	Baseline (mg/dL)	Post-alloxan (mg/dL)	p-value
Non-diabetic group (negative control)	97.4 ± 15.24	104.2 ± 10.27	0.77
Diabetic group (alloxan induction)	94.8 ± 2.05	153.2 ± 2.6	<0.001*

Note: *p <0.05 significantly increased blood glucose in diabetic group

Blood glucose levels were monitored in the experimental groups on Days 7, 14, 21, and 28 following treatment. The negative control group, despite not receiving alloxan, showed a baseline increase in blood glucose from 104.2 mg/dL to 138.6 mg/dL (a 33% increase) over the study period. This observed increase is consistent with the natural metabolic conversion of consumed carbohydrates (monosaccharides, disaccharides, and polysaccharides) into glucose within the liver, a physiological process independent of alloxan induction[36]. In contrast, the alloxan-induced positive control group experienced a substantial elevation in blood glucose, rising from 156.6 mg/dL to 290 mg/dL (an 85% increase), further highlighting the severity of the induced diabetic state.

Remarkably, all three groups treated with *T. erecta* ethanol extract demonstrated a dose-dependent reduction in blood glucose levels from post-alloxan induction (Day 0) to Day 28. Specifically, the 25 mg/kg BW group showed a decrease from 152.8 mg/dL to 122.8 mg/dL (-20%), the 50 mg/kg BW group decreased from 151.8 mg/dL to 115.2 mg/dL (-24%), and the 75 mg/kg BW group exhibited the most pronounced reduction, from 151.6 mg/dL to 97.4 mg/dL (-36%). Statistical

analysis confirmed that the administration of *T. erecta* extract significantly reduced glucose levels in alloxan-induced diabetic rats ($p < 0.05$) (**Figure 1**). These findings align with several previous studies that have also reported the hypoglycemic activity of *T. erecta* (23, 37–39).

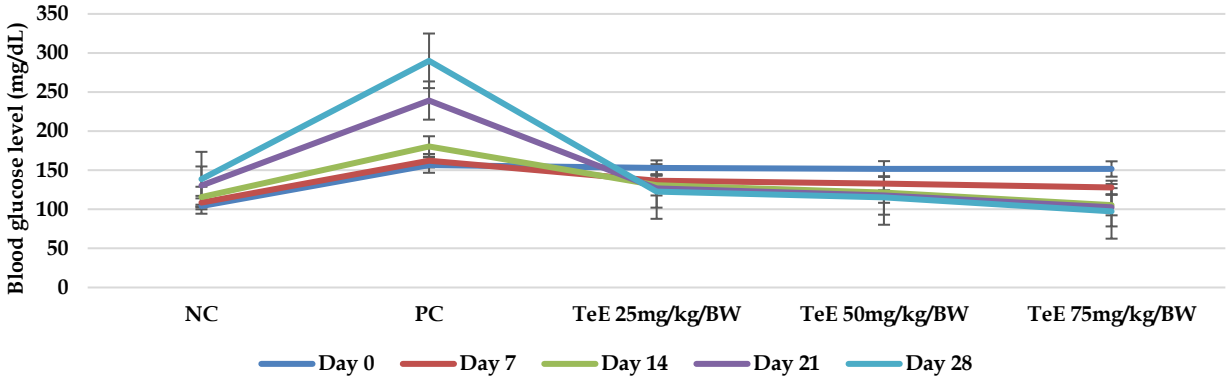

Figure 1. Blood glucose in rats after *T. erecta* treatment. Tamhane test. NC: negative control; PC: positive control; TeE: *T. erecta* extract.

Analysis of TNF-α expression within pancreatic tissue was conducted using immunohistochemical staining, with positive expression visualized as the presence of a brownish precipitate (**Figure 2**). This coloration resulted from the specific binding cascade involving TNF-α, its anti-rat secondary antibody, subsequently an anti-rat IgG, followed by streptavidin-horseradish peroxidase, and finally the DAB chromogen. Quantification revealed a notable increase in TNF-α expression within the alloxan-induced diabetic group compared to the non-diabetic negative control group. Conversely, a reduction in the brownish staining intensity indicated decreased TNF-α expression in pancreatic cells from treated groups. To ensure robust quantification, TNF-α expression was assessed across five distinct visual fields per sample. Statistical comparisons among the various treatment groups were performed using a One-Way ANOVA test, with a confidence level of α=5% (**Table III**).

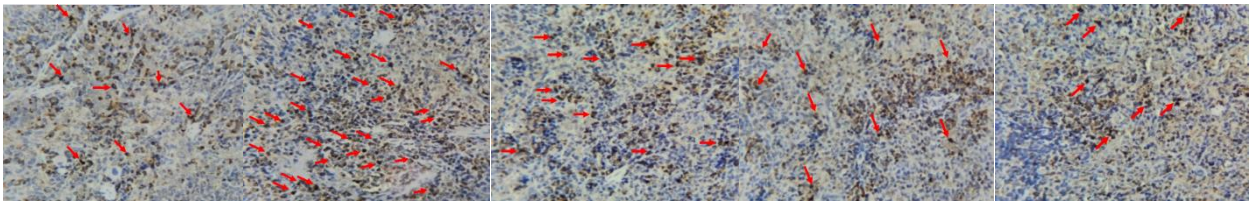

Figure 2. Immunohistochemical staining for pancreatic TNF-α expression after *T. erecta* treatment. Brown staining (red arrows) indicates cells with positive expression (magnification 40x; WD 0.65 mm). (**a**) negative control, (**b**) alloxan-induced, (**c**) *T. erecta* dose 25 mg/kgBW, (**d**) 50 mg/kgBW, and (**e**) 75 mg/kgBW.

Table III. The effect of *T. erecta* on TNF-α expression.

Groups	Mean±SE	p-value
Negative control	474.8 ± 57.27	0.000
Positive control	976.2 ± 49.82	
Tagetes erecta 25 mg/kgBW	635.0 ± 69.44[a]	
Tagetes erecta 50 mg/kgBW	521.6 ± 63.42[a]	
Tagetes erecta 75 mg/kgBW	365.4 ± 15.06[a]	

Note: Tamhane's test [a]$p < 0.05$ compared to alloxan-induced group

Alloxan-induced hyperglycemia significantly increased the production of free radicals, such as nitric oxide radicals, which subsequently led to the accumulation of excessive free radicals. This accumulation, in turn, triggered oxidative stress, causing disruption in the pancreatic β-cells. In a diabetic state, an imbalance between oxidants and antioxidants is indicative of chronic inflammation. Consistent with this, our study observed a high level of TNF-α expression in diabetic rats. These findings align with previous research reporting a prominent increase in TNF-α levels in diabetic animal models[37].

The observed anti-diabetic effects of *T. erecta* are likely attributable to its rich phytochemical composition. Previous research indicates that bioactive compounds such as gallic acid and quercetin are prevalent phenolics, while lutein is a dominant carotenoid in *T. erecta* petals[38]. Specifically, the hypoglycemic effect of *T. erecta* has been linked to its lutein content[23], which may function by inhibiting carbohydrate-degrading enzymes like α-amylase and α-glucosidase, thereby mitigating postprandial hyperglycemia in diabetic rodents[38].

Furthermore, the various classes of metabolites identified in *T. erecta*, including alkaloids, saponins, tannins, and terpenoids, contribute to its antidiabetic potential through diverse mechanisms. Alkaloids, for instance, can interfere with the activity of hydrolase enzymes involved in polysaccharide hydrolysis, consequently reducing glucose formation[39]. Saponins exert hypoglycemic effects by potentially restoring insulin action, promoting insulin release from pancreatic β-cells, inhibiting disaccharide breakdown, stimulating glycogen synthesis, suppressing gluconeogenesis, and enhancing GLUT4 expression[40]. Tannins act as protective agents by scavenging free radicals and activating antioxidant enzymes. They also play a role in increasing glucose absorption via insulin signaling mediators such as Phosphoinositide-3-Kinase (PI3K) and GLUT4 translocation[41]. Terpenoids, isolated from various herbal plants, can improve pancreatic β-cell function, enhance glucose tolerance, and increase the expression of the glucose transporter GLUT4, particularly when insulin function is compromised[42]. Given that impaired GLUT4 function is strongly associated with obesity and diabetes[Error! Reference source not found.], its upregulation by terpenoids is a significant mechanism for maintaining glucose homeostasis. Flavonoid compounds contribute to anti-hyperglycemic effects by binding to peroxisome proliferator-activated receptor gamma (PPARγ) and glucose transporter receptor (GLUT1), thereby stimulating glucose absorption, augmenting insulin action, and improving glucose tolerance in both animal models and humans[42]. PPARγ is crucial for adipogenesis and glucose regulation; its activation, in conjunction with the retinoic acid X receptor, orchestrates the transcriptional activation of downstream target genes associated with diabetes. Additionally, PPARγ is reported to enhance GLUT1 and GLUT4 translocation in the liver and skeletal muscle, and to sensitize insulin by reducing TNF-α and increasing adiponectin expression[43]. Flavonoids also inhibit the activation of nuclear factor kappa B (NF-κB), which is often triggered by excessive free radical production, leading to a reduction in TNF-α expression[37].

Our findings align with previous research demonstrating the anti-inflammatory potential of plant-derived compounds. For instance, a study by Tang *et al*.[44] reported that kaempferide, an active component found in *T. erecta*, effectively reduced serum TNF-α levels in obese mice. Their investigation elucidated a detailed mechanism: kaempferide therapy actively inhibited the activation of Toll-like Receptor 4 (TLR4), concurrently promoting the expression of the inhibitor of NF-κB. This cascade subsequently led to a reduction in the expression of NF-κB, thereby diminishing the levels of pro-inflammatory mediators such as Intercellular Adhesion Molecule 1 (ICAM-1), Vascular Cell Adhesion Molecule 1 (VCAM-1), and TNF-α. Further supporting *T. erecta*'s anti-inflammatory capabilities, studies in non-metabolic inflammatory conditions, such as neuroinflammation, confirmed its ability to reduce the expression of Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), and TNF-α[45]. Additionally, research has shown that *T. erecta* attenuates LPS-stimulated TNF-α production by directly suppressing its mRNA expression[46], highlighting its influence at a transcriptional level.

CONCLUSION

This study demonstrates that *T. erecta* extract exhibits promising antidiabetic and anti-inflammatory properties, evidenced by its significant lowering effect on the pro-inflammatory cytokine TNF-α at the highest tested dose of 75 mg/kgBW. These findings suggest the potential of *T. erecta* as a natural therapeutic agent for managing conditions associated with diabetes and inflammation. However, further investigations employing molecular and cellular approaches are warranted to elucidate the underlying mechanisms responsible for these beneficial effects fully.

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AUTHORS' CONTRIBUTION

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Writing - review & editing: Kadeq Novita Prajawanti, Yohanes Ardian Kapri Negara

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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