ORIGINAL ARTICLE

Effects of Methotrexate, Moringa Leaf (*Moringa oleifera*) Extract, and Sambiloto Leaf (*Andrographis paniculata*) Extract on Blood Glucose Levels, Interleukin-6 Levels, and Trabecular Density in Streptozotocin-Nicotinamide-Induced Hyperglycemic Rodents

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ABSTRACT

Introduction: Methotrexate (MTX), *Moringa oleifera* (MO), and *Andrographis paniculata* (AP) have been reported to have anti-hyperglycemic, antioxidative, and anti-inflammatory effects in diabetic rats. This study aims to investigate the single and combination effects of MTX, MO, and AP on random blood glucose levels, interleukin-6 (IL-6) levels, and trabecular density in diabetic rats. **Methods:** A total of 49 male rats were divided into seven groups, namely one control group and six diabetes mellitus (DM) groups. All rats in the DM groups were injected with streptozotocin-nicotinamide (STZ-NA) intraperitoneally. In addition, the DM groups were administered with a placebo daily (DG), a single dose of 500 mg/kg BW MO daily (DG+MO), a single dose of 500mg/kg BW AP daily (DG+AP), a single dose of 7 mg/kg BW MTX once a week (DG+MTX), a combination of MTX+MO, and a combination MTX+AP, respectively. The experiment lasted for 28 days. On day 29, the right and left femur of the rats were collected for IL-6 examination (ELISA) and histopathological analysis. **Results:** IL-6 expression levels were significantly lower in diabetic rats treated with single and combination of MTX, MO, and AP compared to untreated diabetic rats ($p < 0.05$). However, the random blood glucose levels and trabecular density between treated and untreated diabetic rats were not significantly different ($p < 0.001$, $p = 0.152$). In addition, IL-6 levels were not correlated with trabecular density in all groups ($r = -0.057$, $p = 0.722$). **Conclusion:** Single doses of MTX, MO leaf extract, and AP leaf extract could suppress IL-6 expression in the femur tissue in diabetic rats. However, the IL-6 expression was not correlated with trabecular density although it significantly affected blood glucose levels in this study.
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Keywords: diabetes, bone, IL-6, methotrexate, *Moringa oleifera*, *Andrographis paniculata*

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemia caused by autoimmune disease, insulin resistance, inadequate insulin secretion, or excessive glucagon secretion (1). A fracture is one of the most common complications in DM patients, especially male patients (2). According to the Bone Health and Osteoporosis Foundation, approximately 9.1 million women in the United States suffered from osteoporosis, far outnumbering the estimated 2.8 million men with osteoporosis. Although postmenopausal women are more likely to develop osteoporosis, older men have a higher rate of and suffer from more severe osteoporosis and fractures (3, 4).

Hyperglycemia in DM affects both cellular and extracellular bone matrix. Glucose induces the formation of highly reactive dicarbonyls, which affects the non-enzymatic glycation reaction to produce an irreversible accumulation of advanced glycation end products (AGEs). AGEs stimulate the formation of damaged collagen and reactive oxygen species (ROS), which induce structural changes in the bone through protein modification. Research shows that interleukin-6 (IL-6)

levels in hyperglycemia are associated with the presence of mature functional osteoclasts through modulation of RANKL. An in vitro study showed that the expression of calcitonin receptor and the activity of caspase-3 decreased in hyperglycemia. However, previous studies have not investigated the relationship between these variables in animal models of streptozotocin-nicotinamide-induced hyperglycemia; hence, this study was conducted (5). These processes involve chemical, pro-oxidant, and inflammatory reactions which result in increased oxidative stress, thus impairing organ function. Inflammatory mediators regulated by AGEs and NF-κB-mediated pathways include tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and C-reactive protein (CRP).

Functional changes in osteoblasts and osteoclasts can occur as a result of bone protein modification (6, 7). In type 2 diabetes mellitus, high blood glucose levels can suppress osteoblast formation and increase the apoptosis rate of osteoblasts due to the presence of AGEs. In addition, the apoptosis rate of osteocytes increases in hyperglycemia, causing decreased bone density, including trabecular bone density. Furthermore, prolonged hyperglycemia and increased inflammatory mediators in type 2 diabetes mellitus negatively affect bone metabolism, ultimately leading to bone loss. If both are treated properly, diabetes-induced osteoporosis can be prevented. Currently, the main therapies for osteoporosis do not lower blood sugar levels nor give protection from inflammation. Therefore, it is crucial to find effective substances that have both hypoglycemic and anti-inflammatory effects to treat this condition.

Previous studies have demonstrated the safety of low-dose methotrexate (MTX) as an antidiabetic, anti-inflammatory, and immunosuppressive drug (8, 9). In 2015, the American Diabetes Association (ADA) stated that methotrexate therapy can reduce metabolic risk factors associated with type 2 diabetes mellitus. A study by Pirkmajer reported that the administration of methotrexate can increase blood glucose uptake in skeletal muscles (10).

Recently, herbal alternative medicine has been used to treat several diseases, including DM, due to its antioxidative property that can prevent free radicals from entering the organs. Previous studies have demonstrated that *Moringa oleifera* (MO) leaves and *Andrographis paniculata* (AP) leaves contain compounds that have anti-hyperglycemic, antioxidative, and anti-inflammatory effects, and can lower blood glucose levels (9-12). MO and AP are common plants in tropical and subtropical countries that are rich in nutrients. Therefore, they are consumed as herbal alternative medicine.

Moringa oleifera (MO) contains bioactive compounds including polyphenols, phenolic acids, and flavonoids. One gram of MO leaves contains myricetin (5.6 mg), quercetin (0.2 mg), and kaempferol (7.5 mg), which are classified as flavonoids. In particular, quercetin has a protective effect against β-cell damage because it contains antioxidative, anti-inflammatory, and anti-mutagenic compounds (13). Other studies showed that MO can inhibit osteoclast genesis, osteoblast apoptosis, as well as oxidative stress and inflammatory responses (14).

Andrographis paniculata (AP), as another herbal alternative, contains bioactive compounds including andrographolides, polyphenols, and flavonoids. Andrographolide is a widely investigated compound for its pharmacological activity. In a previous study, Verma et al. demonstrated that AP extract can improve glucose utilization, which prevents hyperglycemia almost as effectively as sulfonylurea therapy (15). AP could inhibit weight loss in diabetic animal models (15). Through inhibiting NF-κB, AP can suppress osteoclast genesis and prevent bone loss caused by inflammation (16).

Based on these explanations, this study aims to investigate the effects of MTX, MO, AP, and their combinations on random blood glucose levels, IL-6 levels, and trabecular bone density in male hyperglycemia rats (Rattus norvegicus) induced with streptozotocin-nicotinamide (STZ-NA). The results of this study may provide basic data on the potential therapeutic property of MTX, MO, and AP as anti-hyperglycemic and anti-inflammatory agents.

MATERIALS AND METHODS

Experimental Animals

This study used an experimental design. The experimental protocols were approved by the Ethics Committee of the Faculty of Medicine, Universitas Airlangga, with a certificate of ethical approval number 1/EC/KEPK/FKUA/2022. This study involved 49 male rats (Rattus norvegicus), aged between two and three months and weighing between 150 and 250 grams. The experiment was conducted in the university laboratory. Upon arrival, all rats were kept in individual cages with standard room temperature (25 ± 2°C), humidity, and controlled lighting (12 hours light and dark) for acclimatization. All rats were fed with standard rodent food (Pokphand CP 593, Charoen Pokphand, Indonesia) and supplied with drinking water ad libitum (17).

After one week of acclimatization, the rats were randomly divided into seven groups, with each group consisting of seven rats. The seven groups were the control group (CG), the diabetic group only (DG), a diabetic group with *Moringa oleifera* leaf extract (DG+MO), a diabetic group with *Andrographis paniculata* leaf extract (DG+AP), a diabetic group with methotrexate (DG+MTX), a diabetic group with a combination of methotrexate and *Moringa oleifera* leaf extract (DG+MTX+MO), and diabetic group with a combination of methotrexate and *Andrographis*

paniculata leaf extract (DG+MTX+AP).

Diabetes Mellitus Animal Models

A single dose of 50 mg/kg body weight (BW) streptozotocin (STZ) and 110 mg/kg BW nicotinamide (NA) were administered intraperitoneally to induce diabetes in the experimental animals. Streptozotocin (BioWorld batch number 41910012-2 and 41910012-3) was dissolved in a citrate buffer (pH 4.5) immediately before injection. Nicotinamide was injected 15 minutes before streptozotocin injection. Nicotinamide can protect pancreatic β-cells from the significant effects of streptozotocin injection (17).

Three days following the injection of STZ-NA, random blood glucose levels were measured using an EasyTouch Glucose Meter (type ET-301F, batch number 301F2C007837, Taiwan). Rats with blood glucose levels above 250 mg/dL were considered diabetic (18).

Treatment

Three days following the intraperitoneal injection, all groups were given oral treatment using a gavage for 28 days. CG and DG were only treated with placebo daily. DG+MO was treated with 500 mg/kg BW *Moringa oleifera* leaf extract daily. DG+AP was treated with 500 mg/kg BW *Andrographis paniculata* leaf extract daily. DG+MTX was treated with 7 mg/kg BW methotrexate once a week. DG+MTX+MO was treated with a combination of methotrexate and *Moringa oleifera* leaf extract, while DG+MTX+AP was treated with a combination of methotrexate and *Andrographis paniculata* leaf extract (19).

This study used commercially available extracts of *Moringa oleifera* (MO) and *Andrographis paniculata* (AP) leaves produced in Indonesia. The MO leaf extract was produced by PT Sido Muncul (7°19′48.7″S 112°45′30.1″E) with a batch number EH00012, while the AP leaf extract was produced by PT Jamu Iboe Jaya (7°22′19.1″S 112°38′40.7″E) with a batch number SB1081A.

Subsequently, the body weights of the rats were measured on days 4 and 28 following the intraperitoneal injection using the Ohauss Triple Beam Balance Set (Smadzu, Japan). Random blood glucose levels were measured on days 3 and 18 following the intraperitoneal injection using EasyTouch Glucometer (type ET-301F, batch number 301F2C007837, Taiwan). Blood samples were obtained using venipuncture of the tail (17).

At the end of the experiment, all rats were sacrificed and the right and left femurs were dissected. Right femurs were fixed in 10% formalin neutral buffer solution to perform histopathological analysis. Meanwhile, the left femurs were fixed in phosphate-buffered saline (PBS) with pH 7.4 to perform the enzyme-linked immunosorbent assay (ELISA) test (20).

Histopathological Analysis of the Femurs

The right femurs of all groups were decalcified in a decalcifying solution (Cal-Ex, Fisher Scientific) for 18 to 24 hours. The bone specimens were dehydrated in an increasing ethanol series before being embedded in paraffin. Tissue paraffin blocks were cut at 5 µm longitudinally parallel to the bone using a microtome. Two sections were used for Mallory-Azan staining. Each section was examined under a light microscope (Olympus CX41) and a digital camera (Olympus DP22) (21).

Furthermore, histopathological analysis was performed to measure the density of the trabecular area in the diaphysis at the distal end of the femur. The analysis was performed using CellSense, Adobe Photoshop, and ImageJ software. Ten visual fields were randomly selected from each slide using an Olympus light microscope and CellSense software with an x200 magnification. The trabecular area of each visual field was blocked using Adobe Photoshop. Subsequently, the density of the trabecular area was measured using ImageJ (22).

Measurement of Interleukin-6 (IL-6) Levels in the Bone Tissue

On day 29, the left femurs of all groups were removed. IL-6 levels were measured using ELISA (Bioenzy, catalog number BZ-08185310-EB, Indonesia). The procedure was replicated in a previous study (20).

Statistical Analysis

Body weight, IL-6 level, and trabecular density values are presented as mean ± SE. Meanwhile, random blood glucose levels are presented as median ± SE. Statistical data analysis was performed using SPPS version 17.0. Comparisons among groups were drawn using the paired t-test for body weight and the Mann-Whitney U test for random blood glucose levels. Moreover, one-way analysis of variance (ANOVA) was used to draw the comparison of IL-6 levels and trabecular density among groups. All tests were considered significant if the p-value was below 0.05 (23).

RESULTS

Single and Combination Effects of MTX, MO Leaf Extract, and AP Leaf Extract on Body Weight

The body weight (BW) of all animals injected with STZ-NA was measured and showed a decrease compared to the control group (not shown here). In this study, the rats' body weight was measured on day 4 and day 28 of the experiment to monitor its modulation (Table I). The body weight of the CG, DG, DG+MO, and DG+MTX groups increased, whereas the body weight of the DG+AP, DG+MTX+MO, and DG+MTX+AP groups decreased following the treatment. Table I shows that DG significantly gained more weight ($p < 0.05$) compared to the other groups. In addition, no significant difference in body weight was observed in the other

Table I. Average body weight (grams) and random blood glucose levels (mg/dL) of the rats

Group	BW			RBG		
	Day 4 (Mean ± SE)	Day 28 (Mean ± SE)	p. value	Day 3 (Median ± SE)	Day 18 (Median ± SE)	p. value
CG	266.71 ± 7.6	281.71 ± 16.46	0.325	108 ± 3.42	105 ± 4.40	0.446
DG	246.43 ± 10.8	282.57 ± 14.79	0.001*	372 ± 20.44	311 ± 40.26	0.310
DG+MO	233.29 ± 8.88	235.43 ± 10.58	0.806	385 ± 38.25	426 ± 33.51	0.735
DG+AP	241.33 ± 9.20	239.17 ± 7.48	0.827	384.5 ± 31.58	449 ± 34.06	0.465
DG+MTX	215.71 ± 7.91	217.71 ± 14.76	0.866	434 ± 35.24	390 ± 24.32	0.735
DG+MTX+MO	232.43 ± 9.34	222.14 ± 14.11	0.496	433 ± 36.64	402 ± 68.83	0.310
DG+MTX+AP	226.67 ± 8.03	212.83 ± 12.23	0.176	420 ± 30.71	413 ± 68.83	0.116

The mean body weight (grams) of male diabetic rat model were measure at day 4 and day 28 after injection STZ-NA. The random blood glucose, which is shown by median value, were measure at day 3 and day 18.
*Significant different (p<0.005).

diabetic groups between day 4 and day 28. On day 28, it was found that the body weight of the DG+MO, DG+AP, DG+MTX, DG+MTX+MO, and DG+MTX+AP groups was less than the control and diabetic groups with significant differences ($p < 0.05$) as shown in Table II. Among the five groups of treated diabetic rats, the differences were not significant ($p > 0.05$) although DG and MTX, either as a single dose or in combination with MO or AP, gained less body weight compared to the groups administered with only MO or AP.

Single and Combination Effects of MTX, MO Leaf Extract, and AP Leaf Extract on Random Blood Glucose Levels

Table I shows random blood glucose (RBG) levels measured on day 3 and day 18. The random blood glucose levels of the CG, DG, DG+MTX, DG+MTX+MO, and DG+MTX+AP groups were lower on day 18 than on day 3, whereas the random blood glucose levels of the DG+MO and DG+AP groups increased from day 3 to day 18. However, no significant difference was observed. On day 18, the control group experienced the lowest random blood sugar level with a significant difference ($p < 0.05$) compared to the other groups (Table III). In addition, it was found that the administration of MTX, either as a single dose or in combination with MO and AP, could not significantly decrease the random blood glucose levels on day 18. On the contrary, the administration of MO and AP extracts to the DG group could increase the random blood glucose level. The follow-up random blood glucose levels should be measured at the end of the experiment to obtain accurate results.

Single and Combination Effects of MTX, MO Leaf Extract, and AP Leaf Extract on IL-6 Levels

IL-6 expression levels at the end of the experiment are presented in Table IV. The one-way ANOVA resulted in a p-value of 0.001 for all groups. The highest mean of IL-6 levels was in the DG group, whereas the lowest mean was in the CG group. Furthermore, the LSD test

Table II: Paired t-test of body weight on day 28 in different groups.

BW DAY 28	CG	DG	DG+MO	DG+AP	DG+MTX	DG+MTX+MO	DG+MTX+AP
CG							
DG	0.964						
DG+MO	0.018*	0.016*					
DG+AP	0.035*	0.031*	0.849				
DG+MTX	0.001*	0.001*	0.349	0.277			
DG+MTX+MO	0.003*	0.002*	0.482	0.387	0.814		
DG+MTX+AP	0.001*	0.001*	0.253	0.200	0.803	0.636	

Mean values of the groups were significantly different.
*Significant different (p<0.005).

Table III: Mann-Whitney U test of random blood glucose levels on day 18 in different groups.

RBG DAY 18	CG	DG	DG+MO	DG+AP	DG+MTX	DG+MTX+MO	DG+MTX+AP
CG							
DG	0.002*						
DG+MO	0.002*	0.249					
DG+AP	0.003*	0.086	0.317				
DG+MTX	0.002*	0.159	0.482	0.317			
DG+MTX+MO	0.035*	0.749	0.949	0.352	0.949		
DG+MTX+AP	0.004*	0.568	0.567	0.337	0.775	1	

Mean values of the groups were significantly different.
*Significant different (p<0.005).

Table IV: IL-6 expression in the femur tissue and trabecular bone density.

Group	IL-6 (Mean ± SE)	p. value	Trabecular bone density (Mean ± SE)	p. value
CG	11.460 ± 0.412		39.514 ± 6.101	
DG	14.070 ± 0.333		27.614 ± 3.567	
DG+MO	12.261 ± 0.426		27.200 ± 3.267	
DG+AP	12.311 ± 0.228	0.001*	29.450 ± 4.128	0.152
DG+MTX	11.516 ± 0.174		26.233 ± 2.752	
DG+MTX+MO	12.520 ± 0.292		26.900 ± 1.652	
DG+MTX+AP	12.778 ± 0.509		26.533 ± 1.709	

The mean values of IL-6 expression are presented in pg/mL. The mean values of trabecular bone density are presented in percentage (%).
*Significant difference ($p < 0.005$)

(Table V) revealed a significant difference between the diabetic group and the control group ($p = 0.000$), which suggested that diabetic rats had an increase in inflammatory mediators. Table V shows that the IL-6 levels were significantly lower ($p < 0.05$) in the DG group administered with either a single dose or a combination of MTX, MO leaf extract, and AP leaf extract compared to untreated diabetic rats.

In addition, it was observed that the IL-6 level of the DG+MTX group was similar to that of the CG group. It is also interesting to note that although the IL-6 levels of the DG+MO and DG+AP groups were higher than that of CG, they were not significantly different ($p > 0.05$). Meanwhile, the IL-6 levels of the DG+MTX+MO and DG+MTX+AP groups showed significant differences compared to the control group ($p < 0.05$). These results suggested that the administration of single doses of MTX, MO leaf extract, and AP leaf extracts were more effective in reducing IL-6 levels compared to the combinations of MTX+MO and MTX+AP in diabetic rats.

Single and Combination Effects of MTX, MO Leaf Extract, and AP Leaf Extract on Trabecular Bone Density

Images of the density of the trabecular area in the diaphysis at the distal end of the femur from seven groups were analyzed using Adobe Photoshop and ImageJ software (Fig. 1). The highest mean of trabecular bone density was seen in the CG group. Table IV shows no statistically significant difference based on the results of one-way ANOVA in the seven groups ($p > 0.05$). This suggested that the administration of a single and combination of MTX, MO leaf extract, and AP leaf extract for 28 days could not prevent bone loss in STZ-NA-induced diabetic rats.

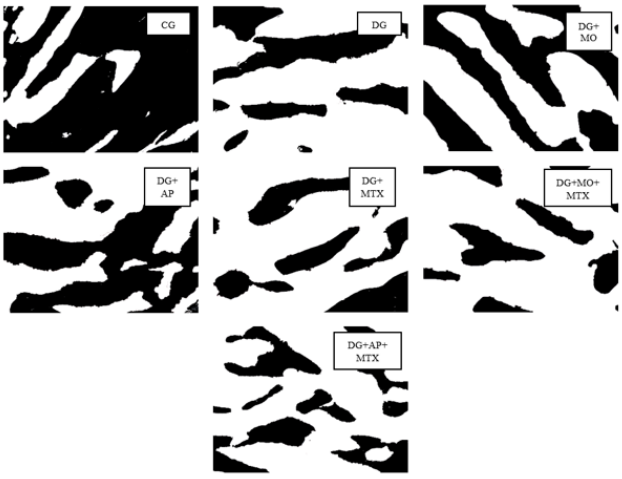

Figure 1: The density of trabecular area. All images show trabecular bone density with an x200 magnification. The analysis was performed using Adobe Photoshop and ImageJ software. The black areas represent trabecular bone, while the white areas represent adipose tissue. Control group (CG); a diabetic group only (DG); a diabetic group with Moringa oleifera leaf extracts (DG+MO); a diabetic group with Andrographis paniculata leaf extracts (DG+AP); a diabetic group with methotrexate (DG+MTX); a diabetic group with a combination of methotrexate and Moringa oleifera leaf extracts (DG+MTX+MO); a diabetic group with a combination of methotrexate and Andrographis paniculata leaf extracts (DG+MTX+AP).

The correlations of random blood glucose levels and IL-6 expression levels with trabecular density are not reported in detail in this study. However, the analysis resulted a in weak correlation ($r = -0,057$, $p = 0.722$).

DISCUSSION

Uncontrolled hyperglycemia can disrupt the function of

Table V: Post hoc LSD of IL-6 expression in the femur tissue.

IL-6	CG	DG	DG+MO	DG+AP	DG+MTX	DG+MTX+MO	DG+MTX+AP
CG							
DG	0.000*						
DG+MO	0.124	0.001*					
DG+AP	0.103	0.001*	0.922				
DG+MTX	0.912	0.000*	0.152	0.127			
DG+MTX+MO	0.045*	0.004*	0.615	0.685	0.057		
DG+MTX+AP	0.014*	0.016*	0.317	0.365	0.018*	0.615	

Mean values of the groups were significantly different.
*Significant different ($p<0.005$).

insulin and hinder the conversion of glucose to energy (7). As a result, the body turns to alternative sources of energy, such as fat and protein, to compensate for this issue, leading to weight loss. In this study, significant differences were observed in the body weight of the groups administered with AP, MTX+MO, and MTX+AP, experiencing weight loss. On the contrary, the groups administered with DG+MTX and DG+MO experienced weight gain. These findings suggested that the administration of MO leaf extract to the experimental animals in this study could promote weight gain (12, 24, 25). Previous studies have demonstrated that the administration of MO leaf extracts can prevent weight loss as a result of the injections of STZ and STZ-NA in diabetic rats (12, 24, 26). Other studies also demonstrated that the presence of antioxidative and antibacterial substances, including phenols, tannins, alkaloids, and quercetins; MO leaf extracts could contribute to its growth-promoting effect on the body weight (12). Furthermore, AP leaf extracts contain polyphenolic compounds that can enhance glucose utilization, similar to the effects of sulfonylurea therapy, thus helping prevent hyperglycemia and inhibit weight loss in experimental animals (15, 25).

Previous studies have suggested that the administration of MO leaf extracts to hyperglycemic animal models resulted in a decrease in blood glucose levels (27, 28). In addition, another study reported that MO leaf extracts have anti-hyperglycemic and antioxidative stress effects (29). Similarly, andrographolides contained in the AP leaf extract were shown to lower blood glucose levels in both diabetic and non-diabetic rats. These compounds enhance the mRNA and protein levels of glucose transporter type 4 (GLUT4), which is the enzyme responsible for transporting glucose across cell membranes, thereby increasing glucose consumption (30). Furthermore, data analysis revealed that the administration of MTX significantly lowered blood sugar levels. The use of MTX treatment has been recommended to reduce metabolic risk factors in DM by the American Diabetes Association (ADA) since 2015. Earlier research also showed that the administration of MTX could enhance muscle glucose uptake (10). Moreover, another study reported that a moderate dose of MTX has an antidiabetic effect (9).

Diabetes mellitus has been reported to cause inflammation of bodily tissues, which is associated with an increase in interleukin-6 (IL-6) levels. Increased IL-6 levels have been found to have implications for bone development based on several investigations. Data analysis revealed that the administration of MO leaf extract to the DG+MO group could reduce IL-6 expression. This aligns with other research findings that showed that quercetin has an anti-inflammatory property, modifying the suppression of the pro-inflammatory cytokine IL-6 (31). As a result, IL-6 levels decrease due to the presence of quercetin in MO leaf extracts (12, 32). Earlier studies have also demonstrated that quercetin contained in MO leaf extract inhibits RANKL-mediated osteoclast genesis, osteoblast apoptosis, oxidative stress, and inflammation (14). Similarly, data analysis revealed that the administration of AP leaf extract to the DG+AP group could reduce IL-6 expression. This aligns with other research findings that showed andrographolide in AP leaf extracts has an anti-inflammatory effect by blocking the NF-κB, AMPK, and PI3K/Akt pathways (33). Another study found that andrographolide as an anti-inflammatory agent suppresses the AMPK signaling pathway, which is the primary instigator of pro-inflammatory cytokines, leading to the inhibition of NF-κB activation and subsequent reduction in IL-6 production (34). Furthermore, this study demonstrated that the administration of a single dose of MTX to diabetic rats could significantly reduce IL-6 levels similar to that of the control group compared to the combination of MTX+MO and MTX+AP. Previous studies have demonstrated that MTX could reduce IL-6 levels in the blood and synoviocytes (35, 36). A comparison between the DG and DG+MTX+AP treatments also revealed significant differences. These findings suggested that the use of the combination treatment decreased IL-6 levels by inhibiting the AMPK and NF-κB pathways (37). It is interesting to note that AP leaf extracts contain andrographolides, which have been shown in another study to have anti-inflammatory properties by blocking the NF-κB, AMPK, and PI3K/Akt pathways (33).

Diabetic conditions pose a serious risk of fractures due to significant loss of bone mineral density. However, according to the results of the data analysis, no significant difference in trabecular bone density was observed in this study. This aligns with other research findings which suggested that hyperglycemia can begin to alter rat bones as early as four to eight weeks, leading to a noticeable reduction in trabeculae within eight to 12 weeks (38). Previous studies revealed that in vivo and in vitro AP extracts can mitigate osteogenesis by inhibiting NF-κb activation, thus preventing bone loss as a result of inflammation (16). On the other hand, long-term treatment with a moderate dose of MTX stimulates the production of osteoclasts, surpassing the activity of osteoblasts and resulting in decreased bone density and permeability (19).

IL-6 plays a role in boosting the production of osteoclasts and NF-κB, which increases the expression of pro-inflammatory mediators TNF-α. As one of the indicators of inflammation in the bone (31), no significant correlation between IL-6 and trabecular density was observed in this study. The findings of this study suggested that although MTX treatment could reduce IL-6 expression, it did not improve bone histology as evidenced by the presence of trabecular density. Therefore, it can be concluded that bone fractures are caused not only by the inflammatory pathway associated with IL-6. In this case, oxidative stress mechanisms could also contribute to trabecular

destruction (39). Furthermore, it may be the case that additional inflammatory indicators, such as CRP and TNF-α, are involved in the process of trabecular bone fractures (40).

CONCLUSION

The administration of methotrexate and *Moringa oleifera* leaf extract could inhibit weight loss in hyperglycemic animal models. However, methotrexate, *Moringa oleifera* leaf extract, and *Andrographis paniculata* leaf extract could not lower random blood glucose levels. Single doses of methotrexate, *Moringa oleifera* leaf extracts, and *Andrographis paniculata* leaf extracts were more effective in decreasing IL-6 expression levels, thus alleviating bone inflammation, although this did not correlate significantly with trabecular density.

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