© 2024 Journal of Pharmacy & Pharmacognosy Research, 12 (2), 382-390, 2024
ISSN 0719-4250
https://jppres.com

DOI: https://doi.org/10.56499/jppres23.1821_12.2.382

Original Article

Effectiveness of *Artocarpus altilis* (Parkinson ex F.A.Zorn) Fosberg ethanol extract in suppressing inflammation and cartilage degeneration in animal models of osteoarthritis

[Eficacia del extracto etanólico de *Artocarpus altilis* (Parkinson ex F.A.Zorn) Fosberg en la supresión de la inflamación y la degeneración del cartílago en modelos animales de osteoartritis]

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Abstract

Context: Osteoarthritis (OA) is a degenerative joint disease characterized by an inflammatory reaction and cartilage degradation. *Artocarpus altilis* is a well-known traditional medicine with various phenolic and flavonoid content. Several pharmacological properties of *A. altilis* extract (AAE) have been reported, but its effectiveness in osteoarthritis is unknown.

Aims: To investigate the potential of AAE as an anti-osteoarthritis.

Methods: The Wistar rats induced OA with mono-iodoacetate (MIA). The OA model rats were treated with AAE with three dose levels (100, 200, and 400 mg/kg) for 28 days. As a positive control used Na-diclofenac (20 mg/kg). Serum IL-1β, IL-10, MMP-3, and MMP-13 levels and histopathological profiles were compared to the MIA group and normal control (5% Na-CMC).

Results: The experimental results showed that AAE could significantly suppress the production of inflammatory mediator factor IL-1β, matrix-degrading proteinase MMP-3, MMP-13, and cartilage degeneration. In addition, AAE increased the production of the anti-inflammatory mediator IL-10, improved synovial inflammation, and increased cartilage thickness.

Conclusions: *A. altilis* extract has the potential to be developed as an anti-osteoarthritis therapeutic agent from natural medicine.

Keywords: *Artocarpus altilis;* cartilage degeneration; inflammatory; mono-iodoacetate; osteoarthritis.

Resumen

Contexto: La osteoartritis (OA) es una enfermedad degenerativa de las articulaciones caracterizada por una reacción inflamatoria y la degradación del cartílago. El *Artocarpus altilis* es una medicina tradicional muy conocida por su contenido en diversos fenoles y flavonoides. Se han descrito varias propiedades farmacológicas del extracto de *A. altilis* (AAE), pero se desconoce su eficacia en la osteoartritis.

Objetivos: Investigar el potencial del AAE como antiosteoartrítico.

Métodos: A las ratas Wistar se les indujo OA con mono-iodoacetato (MIA). Las ratas modelo de OA fueron tratadas con AAE con tres niveles de dosis (100, 200 y 400 mg/kg) durante 28 días. Como control positivo se utilizó Na-diclofenaco (20 mg/kg). Se compararon los niveles séricos de IL-1β, IL-10, MMP-3 y MMP-13 y los perfiles histopatológicos con el grupo MIA y el control normal (5% Na-CMC).

Resultados: Los resultados experimentales mostraron que AAE podría suprimir significativamente la producción del factor mediador inflamatorio IL-1β, la proteinasa degradadora de la matriz MMP-3, MMP-13, y la degeneración del cartílago. Además, la AAE aumentó la producción del mediador anti-inflamatorio IL-10, mejoró la inflamación sinovial y aumentó el grosor del cartílago.

Conclusiones: El extracto de *A. altilis* tiene potencial para ser desarrollado como agente terapéutico anti-osteoartritis desde la medicina natural.

Palabras Clave: *Artocarpus altilis*; degeneración del cartílago; inflamatorio; monoyodoacetato; osteoartritis.

ARTICLE INFO
Received: September 21, 2023.
Accepted: December 7, 2023.
Available Online: January 7, 2024.

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INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease that causes chronic pain, stiffness, and degenerative changes in the articular cartilage in the knees, feet, hands, and hips (Liu et al., 2022; Seo et al., 2022). The incidence of OA increases with age and exceeds 40% in people over 70 years (Cadet et al., 2021; Li and Zhang, 2020; Pérez-Lozano et al., 2021). Even though it is not fatal, OA disrupts the patient's activities and becomes an economic burden for the family (Asante-Kwatia et al., 2020). Pain is one of the most important features of clinical symptoms of OA (Kakatum et al., 2021). OA therapy reduces pain to improve joint function and the patient's quality of life (Chun et al., 2016). The administration of non-steroidal anti-inflammatory drugs (NSAIDs) is the first line of pharmacological therapy for OA (Seo et al., 2022). This drug is symptomatic; it only relieves pain but does not prevent cartilage degradation or repair damage to the cartilage (Ansari et al., 2020). In addition, these agents have adverse side effects and toxicity, such as gastrointestinal ulceration, renal failure, and cardiovascular disorders. These side effects limit treatment options, especially for elderly patients (Cadet et al., 2021; Lee et al., 2019a). Therefore, searching for alternative and effective therapies is necessary to minimize the risk of side effects. Plants in medicine are increasingly attracting much attention because of their wide acceptance, availability, effectiveness, and safety (Asante-Kwatia et al., 2020).

Previously, OA was considered a degenerative joint disease, but recent studies suggest a correlation between the pathogenesis of OA and the inflammatory response and oxidative stress (Park et al., 2017). Therefore, reducing oxidative damage and decreasing inflammation are believed to be beneficial in managing OA. Inflammation and lesions in the knee can be inhibited by anti-inflammatory activity, which suppresses the production of inflammatory mediators (Ho et al., 2019; Huang et al., 2019; Park et al., 2016). Another study states that plants play a role in reducing inflammation and further cartilage damage (Chun et al., 2016).

One tropical plant with the potential as an OA drug is *Artocarpus altilis* (family *Moraceae*), known as Sukun in Indonesia. Several pharmacological properties of *A. altilis* leaves have been reported previously, including antihypercholesterol, antiplatelet, anti-melanogenesis, anticancer, anti-diabetic, kidney disorders, anti-malarial, anti-ulcer and diuretic (Fitrya et al., 2022; 2023b). However, there have been no reports regarding the potential of *A. altilis* leaf extract for its effectiveness as an anti-osteoarthritis. *A. altilis* leaf ethanol extract has also been shown to be an active antioxidant and anti-inflammatory (Palupi et al., 2020; Riasari et al., 2019). Both activities play an important role in overcoming osteoarthritis (Li and Zhang, 2020). Antioxidant activity protects against oxidative damage and is useful for cell regeneration (Huang et al., 2019). Anti-inflammatory activity is important to suppress the production of pro-inflammatory biomarkers that trigger matrix degradation and exacerbate the development of OA (Huang et al., 2019). This study was designed to evaluate the effectiveness of *A. altilis* leaf ethanol extract in overcoming inflammation and cartilage damage in animal models of OA.

MATERIAL AND METHODS

Chemicals and plant materials

A. altilis leaf ethanol extract (AAE) used in this study is part of previous research (Fitrya et al., 2022). The plant specimen vouchers were identified at the Technical Implementation Unit of the Plant Conversion Center of Purwodadi Botanical Gardens – Indonesian Institute of Sciences (LIPI) (No. 165/IPH.6/HM/I/2019) (GPS coordinates 3°12'8.60"S 104°35'36.34"E). Chemicals, including ketamine, xylazine, monoiodoacetate, hematoxylin, and eosin, were obtained from Sigma Aldrigh (Singapore).

Antiosteoarthritis activity test

Sprague-Dawley male rats (7-8 weeks, body weight 190–210 g) were habituated for one week at room conditions with a temperature of 22°C, 55.5% humidity, and 12 hours of light. Eat and drink *ad libitum*. After the habituation period, the rats were randomly divided into six groups: the normal control group (Na-CMC 5%), Mono-iodoacetate (MIA), Na-diclofenac, and three test groups (AAE doses of 100, 200, and 400 mg/kg). All animals (except the normal group) were anesthetized with a mixture of ketamine (25 mg/0.5 mL) and xylazine (20 mg/0.2 mL) intra-muscular. Under anesthesia conditions, rats were induced OA with MIA (3 mg/50 µL 0.9% saline) in the intra-articular cavity of the left knee. Two weeks after induction, the positive group was treated with 20 mg/kg Na diclofenac orally, and the test group was treated with AAE once a day for 28 days orally. On the 29th day, blood was collected from the retroorbital vein of the eye. Blood samples were centrifuged at 2000 rpm for 10 minutes at 4°C, and serum was collected and stored at -80°C. The IL-1β, IL-10, MMP-3, and MMP-13 levels were analyzed by ELISA assay method according to the manufacturer

(Bioassay Technology Laboratory, China) (Lee et al., 2019b; Mohammadifar et al., 2021). All samples were tested in triplicate. After blood collection, animals were euthanized by inhalation using petroleum ether. Then, surgery was performed on the knee to display intra-articular tissue, which was used for histopathological observation. Ahmad Dahlan University Ethics Committee No. 022211088 has approved the animal treatment procedure.

Histopathological analysis

Tissue specimens from rat knee joints were fixed in 10% formalin. Then decalcified in 8% HCl. Then, it was processed into blocks in paraffin and cut using a microtome with a thickness of 4 µm. Tissue preparations were stained with hematoxylin and eosin (H&E). Histological changes were examined using a light microscope (CX-33, Japan) (Endrinaldi et al., 2022). Cartilage thickness was assessed using a 3.1MP Sony Exmor camera (Japan). The thickness of non-calcified cartilage components, calcified cartilage, and total cartilage thickness were measured at 100× microscopic magnification and reported as a mean value of µm in the Beta View program. Histological parameters were assessed semiquantitatively in the form of changes related to osteoarthritis for damage to the cartilage genu according to the OARSI scoring system (Glasson et al., 2010). The assessment of synovitis in osteoarthritis uses criteria based on a semiquantitative scoring system by Takahashi et al. (2018).

Data analysis

All data were analyzed using Statistical Package for Social Sciences (SPSS) version 22.0. Data are presented as mean ± SD. Testing the normality of data distribution using Shapiro-Wilk analysis. The data is normally distributed if the value of $p>0.05$ with a 95% confidence value. Furthermore, normally distributed data were tested with one-way ANOVA and continued with Tukey's post-hoc test to determine differences between test groups ($\alpha = 0.05$).

RESULTS

Effects of extracts on serum cytokine and metalloproteinase levels

Inflammation response is closely related to the pathogenesis of OA; therefore, this study examined pro-inflammatory and anti-inflammatory cytokine levels. The results of the analysis showed that AAE decreased serum IL-1β levels depending on dose (Fig. 1). At an amount of 400 mg, the AAE showed no different effect than the Na-diclofenac group and the normal control group ($p>0.05$). In contrast, the 100 mg dose did not significantly impact because it was not different from the MIA group ($p>0.05$). Conversely, increasing the dose of AAE can increase the production of IL-10. The effect of AAE at doses of 200 and 400 mg, which increased IL-10 production, was no different from the control normal and Na-diclofenac group ($p>0.05$) (Fig. 1).

Increased IL-1β levels led to significant increases in serum MMP-3 and MMP-13 levels of MIA-treated rats and were significantly different from the normal group ($p<0.05$) (Fig. 1). It indicates that induction with MIA causes OA in experimental animals. In contrast, the AAE-treated animals showed a decrease in MMP-3 and MMP-13 levels in line with increasing doses. The 400 mg dose AAE group showed the ability to suppress MMP-3 and MMP-13 production, which was no different from the normal control and Na-diclofenac groups ($p>0.05$).

Effect of extract on the repair of damaged knee tissue

Photomicrograph results of the normal control group showed the knee joint with an entire joint surface, without signs of erosion or destruction. The synovial connective tissue and infrapatellar fat-pad consist of connective and adipose tissue without signs of inflammation. Animals that received MIA induction showed signs of joint damage and synovial abnormalities, joints with irregular surfaces, erosion/destruction of cartilage with decreased chondroblast density, and cartilage thinning. Treatment with AAE showed an improvement in the histological profile of the joint, with a decrease in inflammation and an improvement in the integrity of the joint cartilage and the thickness of the joint cartilage. Treatment with AAE 400 mg is closest to standard drug control (Fig. 2).

Animals that received AAE treatment showed a decrease in signs of joint cartilage destruction; the thickness of the cartilage was higher than the MIA group ($p<0.05$). The AAE 200 and 400 mg groups showed no difference in total cartilage thickness from the normal control and Na-diclofenac ($p>0.05$) (Table 1).

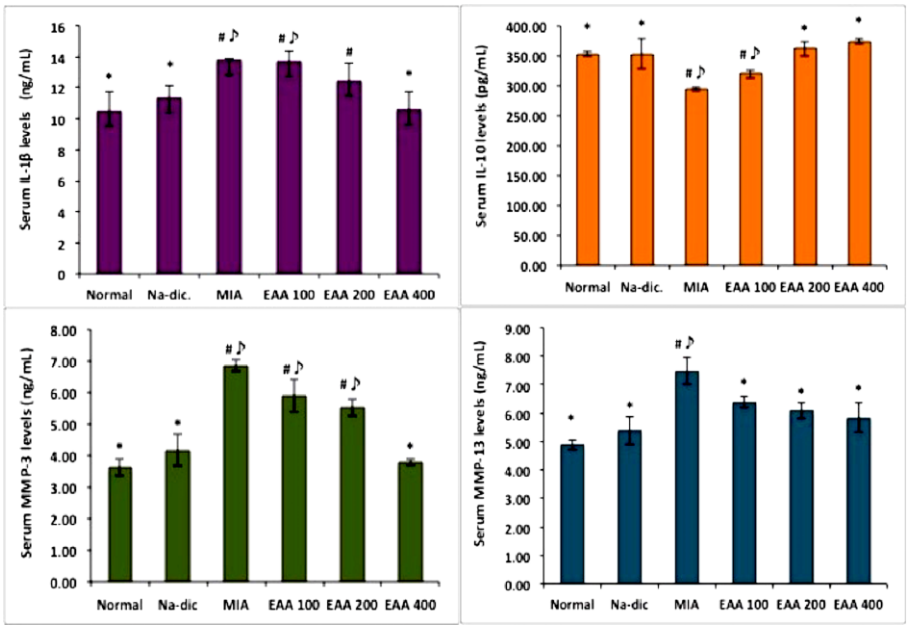

Figure 1. The effect of AAE on OA rats after 28 days of treatment, decreasing serum IL-1β, MMP-3, and MMP-13 levels and increasing IL-10 in rats were measured by ELISA kits.

Data are expressed as mean ± SD (n = 3) and were analysed by one-way ANOVA followed by Tukey's post-hoc test. *p<0.05 compared to MIA; #p<0.05 compared to control normal and ♪p<0.05 compare to Na-diclofenac.

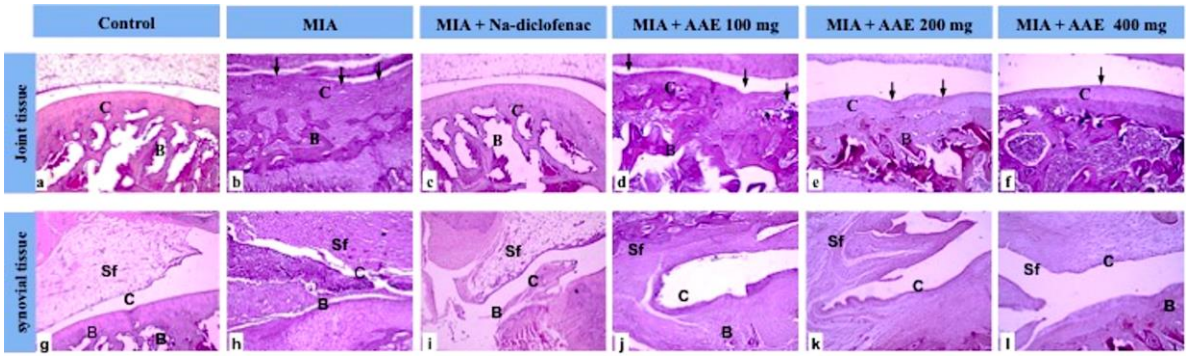

Figure 2. Photomicrograph of joint and synovium infrapatellar fat of animal model OA treated with AAE: joint histology (a-f), synovial and infrapatellar tissue histology (g-l), genu joint cartilage tissue (C), epiphyseal bone (B), synovial and infrapatellar fat pad (Sf), joint surface erosion (↓), Hematoxilyn-Eosin staining (magnification 100×).

Table 1. The effect of *A. altilis* extract on the histological parameter assessment of joint tissue in rats.

Group	The thickness of non-calcified cartilage layer (µm)	The thickness of the calcified cartilage layer (µm)	The total thickness of the cartilage layer (µm)	Joint cartilage damage score	Inflammation score
Control	127.75 ± 4.21*	112.88 ± 20.27*	240.64 ± 16.25*	0	0
MIA	51.71 ± 8.35#♪	51.3 ± 10.70#	103.01 ± 15.59#♪	5.67	3.00
MIA + Na-diclofenac	150.07 ± 15.73*	95.06 ± 10.61	245.13 ± 71.28*	2.33	0.38
MIA + AAE 100	66.71 ± 26.18#♪	103.37 ± 33.23	170.08 ± 34.23♪	4.33	2.00
MIA + AAE 200	92.72 ± 15.87♪	133.22 ± 47.25*	225.94 ± 39.84*	3.67	2.00
MIA + AAE 400	95.28 ± 18.46*	141.16 ± 36.15*	236.44 ± 35.44*	3.00	1.00

Data are expressed as mean ± SD (n = 3) and were analysed by one-way ANOVA followed by Tukey's post-hoc test. *p<0.05 compared to monoiodoacetate (MIA); #p<0.05 compared to control normal and ♪p<0.05 compared to Na-diclofenac.

DISCUSSION

Osteoarthritis (OA) is a degenerative joint disease that causes chronic pain, stiffness, and progressive loss of articular cartilage (Seo et al., 2022). In the pathophysiology of OA, there is a close relationship between inflammation and cartilage degradation. Inflammatory mediators play an important role in causing chronic inflammation and tissue damage during the development of OA because the excretion of pro-inflammatory cytokines will induce matrix-degrading enzymes such as MMP-3 and MMP-13 (Lee et al., 2019b). Therefore, reducing inflammation is the main goal of therapy so that the development of OA can be suppressed (Lee et al., 2019a).

Cartilage degeneration can be induced by administering mono-iodoacetate (MIA). MIA works by inhibiting glyceraldehyde-3-phosphate dehydrogenase, thereby inducing chondrocyte death. MIA injection in animal models will cause cartilage erosion, similar to human OA (Guzman et al., 2003; Takahashi et al., 2018; Xu et al., 2020). The choice of MIA dose was based on previous reports showing that MIA 3 mg can induce joint degeneration, produce significant axonal injury, and reproduce the pain component normally observed in the late stages of OA development (Park et al., 2020).

During the clinical development of OA, pro-inflammatory cytokine levels increase, such as tumor necrosis factor-alpha (TNF-α) and interleukins (IL-1β, IL-6, IL-15, and IL-17) (Ho et al., 2019). The cytokine that plays the most role in the pathophysiology of OA is IL-1β, so it is considered a key cytokine in the pathogenesis of OA (Liu *et al.*, 2022; Pal and Dhobi, 2019). This cytokine potentiates extracellular matrix (ECM) degradation and chondrocyte apoptosis (Ho et al., 2019; Liu et al., 2022). The results of this study indicated that animals that received MIA injections experienced a significant increase in IL-1β levels compared to normal control animals (Fig. 1). IL-1β is synthesized by chondrocytes, osteoblasts, synovial membrane-forming cells, and mononuclear cells in inflamed joints (Lee et al., 2019a). The binding of IL-1β to IL-1R receptors in synovial membrane cells will activate NF-κB and MAPK signaling pathways that regulate inflammatory responses to produce inflammatory mediators such as MMPs, COX-2, PGE_2, NO, and other catabolic factors, which can accelerate cartilage degeneration. Therefore, blocking the expression of pro-inflammatory cytokines, which will inhibit inflammatory signaling pathways, is a new strategy in OA therapy (Liu et al., 2022). In addition, this increase in cytokines also induces the production of matrix-degrading proteins, including matrix metalloproteinases (MMPs) (Choi et al., 2020). Among these proteinases, MMP-3 and MMP-13 are the proteases that play the most role in degrading matrix and collagen (Imbawan et al., 2011; Zhang et al., 2016). This study proves that the group that received the MIA injection experienced an increase in serum levels of MMP-3 and MMP-13 in line with the rise in IL-1β levels. In contrast, AAE treatment significantly suppressed IL-1β, MMP-3, and MMP-13 production in animal models of osteoarthritis (Fig. 1). This study's results are in line with previously reported (Choi et al., 2020; Park et al., 2017; Zhao et al., 2018).

Matrix metalloproteinases are OA biomarkers tissues release into the blood or synovial fluid. Matrices level changes reflect synovial metabolism and bone or cartilage turnover, so they are often used as pathophysiological parameters (Choi et al., 2020). MMPs are key enzymes in extracellular matrix (ECM) degradation. They can degrade the extracellular matrix's main components at physiological pH (Shiomi et al., 2010). MMP-3 (stromelysin-1) is the main enzyme that plays a major role in the destruction of joint cartilage in osteoarthritis, besides MMP-1, MMP-9, and MMP-13 (Imbawan et al., 2011). MMP-3 damages not only collagen type II but also proteoglycans, collagen types IV and IX, and osteonectin in cartilage. Increased synthesis of these degradative enzymes further exacerbates articular cartilage damage (Sofia et al., 2019). This study showed that animals injected with MIA showed the highest levels of MMP-3 and MMP-13 (Fig. 1) and the most severe synovitis (Fig. 2). The abnormalities observed in proteinase and tissue damage reported in this study are consistent with previous investigations (Lee et al., 2019a; 2019b). In contrast, the group that received Na-diclofenac and AAE 400 mg showed a profile that resembled the normal control group, in line with decreased levels of MMP-3 and MMP-13. MMP has a natural inhibitor, namely Tissue Inhibitor Matrix Metalloproteinases (TIMPs), but because in the case of OA, MMP enzyme activity is dominant, so what happens is cartilage catabolism (Imbawan et al., 2011).

Anti-inflammatory cytokines are required to combat pro-inflammatory cytokines. IL-10 is a macrophage-produced anti-inflammatory cytokine that inhibits the secretion of various pro-inflammatory cytokines (Liu et al., 2022). These cytokines suppress inflammation in the synovial membrane and act as anabolic effectors that can moderate the development of OA. IL-10 shows a chondroprotective effect during the OA range by stimulating the synthesis of type II collagen and aggrecan and being responsible for suppressing the formation of metalloproteinases (Pal and Dhobi, 2019). In addition, IL-10 can also activate the SMAD1/SMAD5/SMAD8 and ERK1/2/MAPK

signaling pathways and induce the expression of bone morphogenetic protein (BMP)-2 and BMP-6. BMP proteins play an important role in chondrogenesis (Liu et al., 2022). It could explain the increase in IL-10 levels in AAE-treated animals and a change in the histological profile of the animal's knee tissue towards normal conditions (Fig. 2).

The ability of AAE to inhibit pro-inflammatory cytokines and cartilage degeneration is related to antioxidant and anti-inflammatory activities reported previously (Fitrya et al., 2023a; Palupi et al., 2020). Antioxidant activity protects against oxidative stress from cellular to organ levels (Fitrya et al., 2022). Increasing cellular antioxidant activity is a way to prevent structural changes in articular cartilage (Teng et al., 2023). It is widely accepted that levels of oxidative stress increase with aging. In cartilage, ECM protein tends to be the main target of ROS (Yagi et al., 2020). ROS can induce replicative aging of chondrocytes. Consumption of antioxidants has been shown to affect the development of OA by eroding ROS molecules, inhibiting pro-oxidant gene expression, and increasing the expression of antioxidant genes such as SODs and catalase (Huang et al., 2019). AAE was reported to be able to increase SOD enzyme levels and reduce H_2O_2 production in *in vivo* experiments (Palupi et al., 2020). H_2O_2 is an oxidant species that can induce chondrocyte cell death (Endrinaldi et al., 2022). Besides H_2O_2, another ROS, namely nitric oxide (NO), is produced by the inflamed synovium, and this molecule changes the balance of cartilage matrix degradation and repair (Lee et al., 2019b). NO increases inflammation by activating the nuclear factor *kappa* B (NFκB) pathway, resulting in increased production of IL-1β and TNFα (Ansari et al., 2020). Palupi et al. (2021) reported that AAE effectively inhibited NO production (IC_{50} = 0.454 ± 0.11 mg/mL), so it can be understood that the ability to reduce the production of the cytokine IL-1β in this study is related to its antioxidant activity.

Polyphenolic compounds' potential OA suppressive activity is related to their anti-inflammatory properties. Polyphenols play a role in suppressing pro-inflammatory signaling pathways such as MAPK (Ansari et al., 2020). The anti-inflammatory effect shown by AAE in this study is consistent with our previous reports that AAE can suppress IL-1β and IL-6 expression and increase IL-10 expression in RAW 64.7 cells (Fitrya et al., 2023a). Fakhrudin et al. (2015) also stated that *A. altilis* extract effectively inhibited COX and was more selective for COX-2 (IC_{50} = 3.17 g/mL) than COX-1 (IC_{50} = 23.45 g/mL). The COX-2 is the main contributor to prostaglandin synthesis.

Phenolic compounds generate anti-inflammatory activity in the synovial by regulating various inflammatory factors, such as MMPs, ILs, and tumor necrosis factor (TNF)-α (Li and Zhang, 2020). Flavonoid compounds suppress the development of OA by reducing the production of NO, PGE2, TNF-α, MMP-2, MMP-3, and MMP-9, which are induced by IL-1β and by inhibiting cartilage degradation and increasing the expression of type II collagen (Pérez-Lozano et al., 2021). Among the flavonoid compounds that have been reported, the compounds quercetin and kaemferol work by reducing inflammation and suppressing the expression of the inflammatory mediator IL-1b (Ji et al., 2023; Shao et al., 2022). Apigenin, on the other hand, reduces the levels of IL-1β, IL-6, and TNF-α while increasing the levels of IL-10 (Ji et al., 2023). Consistent with previous reports, this study found that AAE suppressed the production of the pro-inflammatory cytokine IL-1β and matrix-degrading proteinases MMP-3 and MMP-13. It then increased the production of the anti-inflammatory cytokine IL-10. The ethanol extract of *A. altilis* is reported to have various flavonoid compounds such as geranyl flavonoids (Inoue et al., 2018; Wang et al., 2007), prenylated flavonoids, geranyl aurone and geranyl dihydrochalcones (Nguyen et al., 2014).

Intra-articular injection of MIA induced histopathological changes in the synovial membrane and articular cartilage (fibrillation, erosion, and osteophyte formation) (Takahashi et al., 2018). Histological examination revealed that the MIA injection group had joint damage, irregular joint surfaces with joint surface erosion and signs of destruction, decreased chondroblast density, and cartilage thinning. On the other hand, in animals treated with AAE, there was a significant improvement in the thickness of the joint cartilage (Table 1). Referring to the grade determined by Glasson et al. (2010) and Takahashi et al. (2018), animals that received AAE treatment showed potential for improvement with reduced joint damage and inflammation scores in line with increasing doses. Improvement in the thickness of the articular cartilage is related to the protective effect of suppressing inflammation or the effect of stimulating chondrocyte regeneration. In this study, inflammation of the synovial was reduced. It confirms the anti-inflammatory effect and the possible stimulation of fibrogenesis by AAE.

CONCLUSION

Based on the results of this study, it can be concluded that AAE can inhibit cartilage degradation by suppressing the production of the pro-inflammatory mediator IL-1β and the matrix-degrading enzymes

MMP-3 and MMP-13. Besides that, AAE has the potential to repair joints by increasing the production of anti-inflammatory mediators IL-10 to provide a protective effect on OA cartilage. These findings suggest that AAE could be a useful therapeutic agent for OA patients. More studies are needed to affirm the latter.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

This research was supported by the Directorate of Research, Technology and Community Service, Directorate General of Higher Education, Research and Technology, which has funded this research through the Implementation of the 2023 State University Operational Assistance Program No 164/E5/PG.02.00.PL/2023 and derivative contract 0144.04/UN9/SB3.LP2M.PT/2023. Thank you to Dr. Tofrizal, Ph.D., and the Head of the Department of Anatomy, Faculty of Medicine, Andalas University for his assistance in histopathological analysis.

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AUTHOR CONTRIBUTION:

Contribution	Fitrya	Muharni	Wahyuni FS	Amriani A
Concepts or ideas	x	x	x	x
Design	x	x	x	x
Definition of intellectual content	x	x	x	x
Literature search	x			x
Experimental studies	x		x	x
Data acquisition	x	x	x	x
Data analysis	x	x	x	x
Statistical analysis	x			x
Manuscript preparation	x			
Manuscript editing	x	x	x	x
Manuscript review	x	x	x	x

Citation Format: Fitrya, Muharni, Wahyuni FS, Amriani A (2024) Effectiveness of *Artocarpus altilis* (Parkinson ex F.A.Zorn) Fosberg ethanol extract in suppressing inflammation and cartilage degeneration in animal models of osteoarthritis. J Pharm Pharmacogn Res 12(2): 382–390. https://doi.org/10.56499/jppres23.1821_12.2.382