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Ethanolic extracts of babandotan leaves (*Ageratum conyzoides* L.) prevents inflammation and proteoglycan degradation by inhibiting TNF-α and MMP-9 on osteoarthritis rats induced by monosodium iodoacetate

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ABSTRACT

Objective: To analyze the effects of *Ageratum conyzoides* L. on the monosodium iodoacetate induced osteoarthritis rats.

Methods: Thin layer chromatography was performed to analyze the constituents of the babandotan extract leaves. White male Sprague–Dawley rats used in this study were divided into 6 groups: normal control and negative control groups, both given 0.5% carboxymethyl cellulose; the positive control group that was given glucosamine and chondroitin suspension (486 mg/200 g B.W.); the 3 dose variation extract groups including dose 1, 2, and 3 that were given 40, 80, and 160 mg/200 g B.W. respectively on day 29 until 50. All the groups were induced with 0.05 mL monosodium iodoacetate (20 mg/mL) on day 1, except normal control induced by saline. Measurement of edema volume of rat knees was performed on day 0, 8, 15, 22, 29, 43, and 50. Hematology data was measured at day 1, 29 and 50. Serum was collected at day 50 to evaluate TNF-α and MMP-9 by ELISA. Cartilage histopathology was evaluated by staining with H&E and Safranin-O-fast green staining on day 50.

Results: The babandotan leaves extract dose 2 (80 mg/200 g B.W.) and dose 3 (160 mg/200 g B.W.) could decrease the edema volume, increase the area and thickness of articular cartilage, and increase proteoglycan level. Particularly, dose 3 (160 mg/200 g B.W.) of extract babandotan leaves were able to significantly decrease the number of leukocytes, lymphocytes and udem volume, and decrease TNF alpha and MMP-9 levels.

Conclusions: Babandotan leaves extract can recover inflammation and cartilages degradation by inhibiting TNF-α in inflammation processes and MMP-9 in the collagenase reaction in the cartilages.

1. Introduction

Osteoarthritis (OA) is characterized by joint pain, limited motion, and inflammation without systemic effects [1]. According to the World Health Organization (WHO) data, OA is one of the main causes of malfunctions and reduces the quality of 151 million people's life worldwide and 24 million people's in Southeast Asia [2]. Indonesia ranked the 4th with highest number of elderly people affected with OA after China, India and the United States.

OA is the most common joint disorder in the world, but there is no approved therapeutics to prevent disease progression. Historically, OA has been considered a wear-and-tear joint disease, and efforts to identify and develop disease-modifying therapeutics have been predominantly focused on direct inhibition of cartilage degeneration. However, now there is increasing evidence that inflammation is a key mediator of OA joint pathology, and also the link between obesity and that OA is not solely due to excessive load-bearing, therefore suggesting that targeting inflammation in OA could be a rewarding therapeutic strategy [3]. The proinflammatory cytokines involved in OA, TNF-α and IL1β, are considered the major implicated [3]. TNF-α promotes inflammation in blood vessel walls to induce

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endothelial cell injury, and regulate leukocyte activation, maturation and release of cytokines and chemokines [4].

Cartilage degradation is a central event of OA, driven by an imbalance of metabolic signals and perpetuated by degradative metallo-proteinases (MMP) [5]. MMPs are categorized into the following groups: collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11), matrilysin (MMP-7), metalloelastase (MMP-12), and membrane-typematrix metalloproteinases (MT-MMP-1, 2, 3, and 4) [6]. A direct route into the bloodstream via the subchondral microcirculatory system and an indirect route from synovial fluid into circulation could explain the higher plasma levels of MMP-9 in OA.

OA with symptoms of mild to moderate pain, the first line of therapy to cure this condition is the use of nonsteroidal anti-inflammatory drugs (NSAID). However, the side effects of gastrointestinal and cardiovascular risk of NSAIDs limited long-term use of NSAIDs specifically in geriatric patients. Special attention needs to be recommended when NSAIDs are administered to patients with cardiovascular risk, as well as the use of selective COX-2 inhibitors [7]. Other therapies commonly used are supplements containing glucosamine and chondroitin sulfate [8]. The combination of these supplements are derived from the processing of marine animals, consequently, glucosamine and chondroitin sulfate cannot be consumed by patients with OA who have a history of allergy to marine animals [9]. Because the drugs to treat OA are still limited, this becomes one attention of researchers to find potential drug candidates that can cure OA from natural products.

Babandotan leaves [*Ageratum conyzoides* L (*A. conyzoides* L)] is natural product that has anti-inflammatory effect [10]. Previous research showed anti-inflammatory activity of hydro-alcoholic babandotan leaves extract at the dose of 250–500 mg/kg in rat model of chronic inflammation [11]. The anti-inflammatory activity of babandotan leaves by inhibition of IL-6. Flavonoid glycoside compounds in the extracts of *A. conyzoides* leaves has an important role as an anti-inflammatory compound [12].

In this study, the effects of *A. conyzoides* L. on the monosodium iodoacetate induced OA rats were analyzed to evaluate its effects on TNF-α and MMP-9 which control inflammation and degradation of proteoglycan on the OA.

2. Material and methods

2.1. Chemicals and reagents

Monosodium iodoacetate, ethanol 96%, methanol p.a, ethyl acetate p.a and petroleum ether p.a. were obtained from Sigma (St. Louis, MO, USA). TNF-α ELISA kit (Catalogue No.: ER1393) and MMP-9 ELISA Kit (Catalogue No.: ER139) were purchased from Finetest®. All other reagents were analytical grade.

2.2. Plant materials

A. conyzoides (babandotan) leaves as the main material, was obtained from the Research Institute for Spices and Medicinal Plants, Bogor and determined by the Research Center for Biology, Indonesian Institute of Sciences (Certificate of Determination No. 206/IPH.1.01/If.07/I/2016). This plant was harvested from plants grown in Bogor, Indonesia (Bogor climate: temperature 21.8–30.4 °C/average 26 °C, humidity 70%, rainfall is quite high average 3 500–4 000 mm per year, altitude 190–133 m above sea).

2.3. Preparation of babandotan leaves extract

The dried powder of babandotan leaves (*A. conyzoides* L.) were extracted by maceration method using ethanol. Then the extract was concentrated by rotary evaporator (Eyela, Tokyo Rikakikai, Tokyo, Japan). The extractive value of ethanol from dried powder was calculated as % w/w yield and was found to be 16.2%.

2.4. Identification and quantification of quercetin content of babandotan

A total of 20 mL sample solution (10 mg/mL) was spotted on a silica gel 60 F254 plate using automatic thin layer chromatography (TLC) sampler. The elution process was performed until the eluent reaches the finish line. Eluent composition was toluene: ethyl acetate: format acid (5:4:0.2). The spot then was evaluated by automatic TLC sampler and TLC equipment identity (CAMAG, Switzerland).

2.5. Animals

White male Sprague–Dawley rats, aged 30 d, were purchased from Indonesia National Institute of Health Research and Development. The animals were grouped and housed in poly-acrylic cages and maintained under standard laboratory conditions [temperature (25 ± 2) °C] with dark and light cycle (12/12 h) and allowed free access to commercial pellet diet and water *ad libitum*. This research had been certified by ethical certification of Faculty of Medicine, University of Indonesia (UI FK No. 75/UN2.F1/Ethics/2016) for the use of animals in experiments.

These Sprague–Dawley rats were divided into 6 groups: normal control and negative control groups, both given 0.5% carboxymethyl cellulose; the positive control group, given glucosamine and chondroitin suspension (486 mg/200 g B.W.); and 3 dose variation extract groups including dose 1, 2 and 3 that were given 40, 80, and 160 mg/200 g B.W. respectively on day 29 until 50. All the groups were induced with 0.05 mL monosodium iodoacetate (20 mg/mL) on day 1, except normal control induced by saline.

Rats were induced by 50 μL monosodium iodoacetate (20 mg/mL) by intraarticular injection; furthermore, three doses of extracts of babandotan leaves were administrated on day 29 until day 49 after monosodium iodoacetate injection. The edema was evaluated every week.

2.6. Hematology analysis

Hematology data were taken on day 0, 29 and 50 and blood were collected from orbital sinus and analyzed by Haematology Analyser (Medonic, Sweden).

2.7. Detection of TNF-α and MMP-9 levels

Blood samples were collected at day 50 and then centrifuged at 3 000 rpm for 15 min to get serum and keep in −30 °C until

analyzed. TNF-α and MMP-9 were determined by ELISA according to the manufacturer's instructions, and read the absorbance using microplate reader (Biochrom, Holliston, USA).

2.8. Histopathological investigation of knee joint

Histology data was conducted after sacrificed the rats and isolated the knee joint. After kinds of histology procedure to prepare isolated knee joint to become slice of knee joint, then the slices were stained by hematoxylin and eosin (H&E) and safranin O- fastgreen. The histological changes were observed under laser scanning microscopy (Olympus, Tokyo, Japan).

2.9. Statistical analysis

Statistics software (SPSS version 16.0) was used for statistical analysis. The data represented mean ± standard error of the mean. Statistical calculations were analyzed by One-way ANOVA followed by multiple comparison tests. *P* value < 0.05 were considered to be significant (*P* denoted probability).

3. Results

3.1. Evaluation of quercetin content of babandotan leaves extract

Figure 1 showed that the extract contained quercetin that showed the same spot at retention factor (Rf) 0.51 compared to the standard quercetin. Furthermore, the concentration of quercetin was calculated and it was found that the concentration was (52.71 ± 2.21) ppm.

3.2. Edema profile

Edema volume measurement was done on day 0, 8, 15, 22, 29, 36, 43 and 50. Measurements were made to determine changes in the volume of edema in animals that were given the extract as shown in Figure 2. It showed that injection of monosodium iodoacetate on rat knee edema significantly increased the volume of edema in all groups given by induction of monosodium iodoacetate on day 8 to day 29. Starting from 14 d after administration of the extract, there were significant differences between the negative control and the dose 2 ($P = 0.013$) and dose 3 ($P = 0.002$). This indicated that the extract doses of 2 and 3 could reduce the rat knee edema volume 14 dafter administration of the extract.

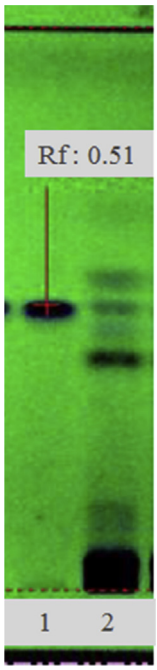

Figure 1. TLC of babandotan leaves extracts.
(1): 100 ppm of quercetin standard; (2): 10 mg/mL extracts.

3.3. Hematology

3.3.1. Hematology evaluation after monosodium iodoacetate treatment

Table 1 showed that, monosodium iodoacetate treatment could increase the number of white blood cell (WBC), lymphocyte but have no effects on granulocytes cells in all groups. Twenty-eight days after administration of monosodium iodoacetate, the leukocytes of all groups which were treated with the iodoacetate showed higher number of leukocytes compared with normal group. There were significant differences between normal group with all other group, negative group ($P = 0.001$), positive group ($P = 0.002$), dose 1 ($P = 0.000$), dose 2 ($P = 0.005$) and dose 3 ($P = 0.000$). Similar results were found in lymphocyte: there was significantly more lymphocyte in normal group compared with negative group ($P = 0.004$), positive group ($P = 0.014$), dose 1 ($P = 0.016$), dose 2 ($P = 0.037$) and dose 3 ($P = 0.015$), with the number of lymphocytes lowest averages were in the normal control group. These results indicated monosodium iodoacetate could make changes in the immune system of osteoarthritis model rats.

3.3.2. Hematology evaluation after babandotan leaves extract treatment

Table 2 showed that on 21 d after administration of the extract, the leukocyte count of negative group were still significantly higher than that of normal group ($P = 0.040$). The administration of the extract could reduce the number of leukocytes as shown in Table 2. The number of leukocytes in positive group and dose 3 were significantly lower than that in the negative group ($P = 0.015$; $P = 0.002$ respectively). It was also found that dose 3 statistically reduced more leukocyte number than dose 1 ($P = 0.024$). Similar results also were also shown at the lymphocytes calculation 21 d after extract treatment.

3.4. Histology evaluation

3.4.1. H&E staining

Figure 3 showed that normal group had a smooth surface and flat and regular arrangement of chondrocytes in the superficial zone and the middle (middle zone) of articular cartilage, while on the articular cartilage of negative control (Figure 3B), cartilage became thinner and uneven surfaces due to their fibrillation (black arrow). There was also a vertical crack that disrupted cartilage matrix components (blue arrows). On the positive control preparations (Figure 3C) which was given the suspension of glucosamine, articular cartilage surface (black arrow) were smooth and flat and also had arranged regularly chondrocytes (blue arrows). The administration of babandotan leaves extract in all doses showed the flat and smooth cartilage surface

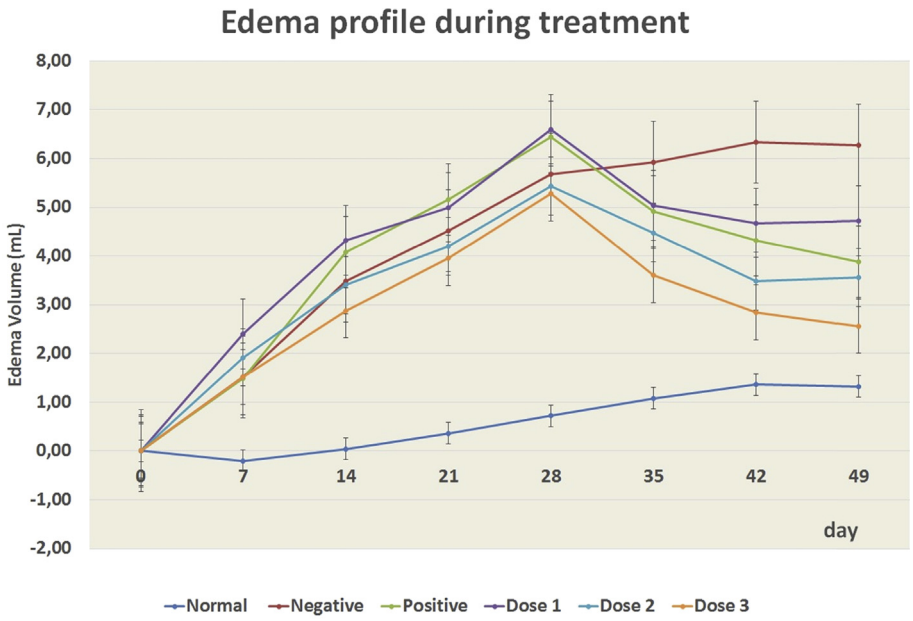

Figure 2. Edema profile during treatment.

Table 1

Number of white blood cells (WBC), lymphocyte (Lym) and granulocytes (GRAN) ($\times 10^9$/L) after induction with monosodium iodoacetate.

Group	WBC	LYM	GRAN
Normal control	9.56 ± 0.58	6.60 ± 0.93	1.70 ± 0.96
Negative control	15.52 ± 1.42*	10.70 ± 2.28*	3.00 ± 1.39
Positive control	14.84 ± 1.75*	10.52 ± 1.84*	2.88 ± 0.90
Dose 1	16.00 ± 1.54*	11.64 ± 3.25*	2.98 ± 2.68
Dose 2	15.60 ± 1.08*	11.56 ± 2.73*	3.00 ± 2.42
Dose 3	13.72 ± 0.79*	9.06 ± 2.72*	3.66 ± 2.40

*$P < 0.05$ as compared to normal control group.

and the chondrocytes were appeared in the superficial part. In dose 3 (160 mg/200 g B.W.), the cartilage surface was smooth and flat and had a full matrix with chondrocytes were arranged regularly.

Furthermore, the thickness and the area of articular cartilage in H&E staining preparate were evaluated. This evaluation was done to find out the effect of babandotan leaves extract on the integrity of the cartilage matrix of OA rats as shown in Table 2. The articular cartilage of negative group were significantly thinner than that of the normal group ($P = 0.002$). It was also found that positive group, dose 2 and dose 3 were all significantly thicker than negative group ($P = 0.000$; $P = 0.001$; $P = 0.000$ respectively).

Besides the thickness of articular cartilage, the cartilage area of OA rats was also evaluated as shown on Table 2. The cartilage area of dose 1 was statistically lower than that in dose 3 ($P = 0.000$) and similar result was shown between dose 2 and dose 3 ($P = 0.025$). Observation of the area and thickness of cartilage showed synergistic results that the positive control and the babandotan leaves extract with dose 2 and dose 3 were able to increase the value of the area and thickness of the articular cartilage significantly. It showed that the integrity of the cartilage matrix improved after being given treatment. Dose 3 (160 mg/200 g B.W.) also showed better results compared with positive control.

3.4.2. Safranin-O-fast green staining

In addition to H&E staining, we also performed special histochemical staining using safranin-O-fast green. Dose 1 was not able to repair the damage of articular cartilage matrix so that the amount of proteoglycans in dose 1 was not significantly different with the negative control. Based on the results on Figure 4, there were differences in the intensity of the red color

Table 2

Hematological index ($\times 10^9$/L), articular cartilage [area (μm^2)], color intensity, and serum index (pg/mL) after babandotan extract treatment.

Group	Hematological index			Articular cartilage		Color intensity	Serum index	
	WBC	Lym	GRAN	Average of cartilage area	Thickness of cartilage		TNF-α	MMP-9
Normal control	11.70 ± 2.33#	7.88 ± 1.55	2.56 ± 2.76	14515.76 ± 2977.25	91.85 ± 12.66	131.47 ± 8.85#	215.01 ± 0.05#	4.012 ± 0.030#
Negative control	18.00 ± 3.27	11.68 ± 3.39	4.36 ± 2.97	8225.17 ± 693.84*	55.68 ± 9.43	168.07 ± 8.00*	7494.70 ± 0.13*	78.070 ± 0.427*
Positive control	11.36 ± 3.97#	7.38 ± 3.35	3.30 ± 1.07	17014.20 ± 3138.22#	108.74 ± 16.76#	127.13 ± 6.36#	913.80 ± 0.01#	3.918 ± 0.056#
Dose 1	13.12 ± 3.21	9.88 ± 2.53	2.32 ± 1.31	12702.15 ± 3279.03	75.36 ± 11.19	157.21 ± 1.71	936.84 ± 0.01#	13.216 ± 0.032#
Dose 2	12.40 ± 2.29	8.12 ± 2.57	2.34 ± 1.28	13515.54 ± 1807.40#	95.23 ± 14,65#	136.64 ± 13.46	1336.15 ± 0.03#	5.617 ± 0.028#
Dose 3	9.66 ± 2.55#	5.80 ± 2.96#	2.94 ± 0.81	18376.71 ± 2244.13#	122.56 ± 9.91#	124.40 ± 13.93#	598.968 ± 0.06#	3.162 ± 0.061#

*$P < 0.05$ as compared to normal control group; #$P < 0.05$ as compared to negative control group.

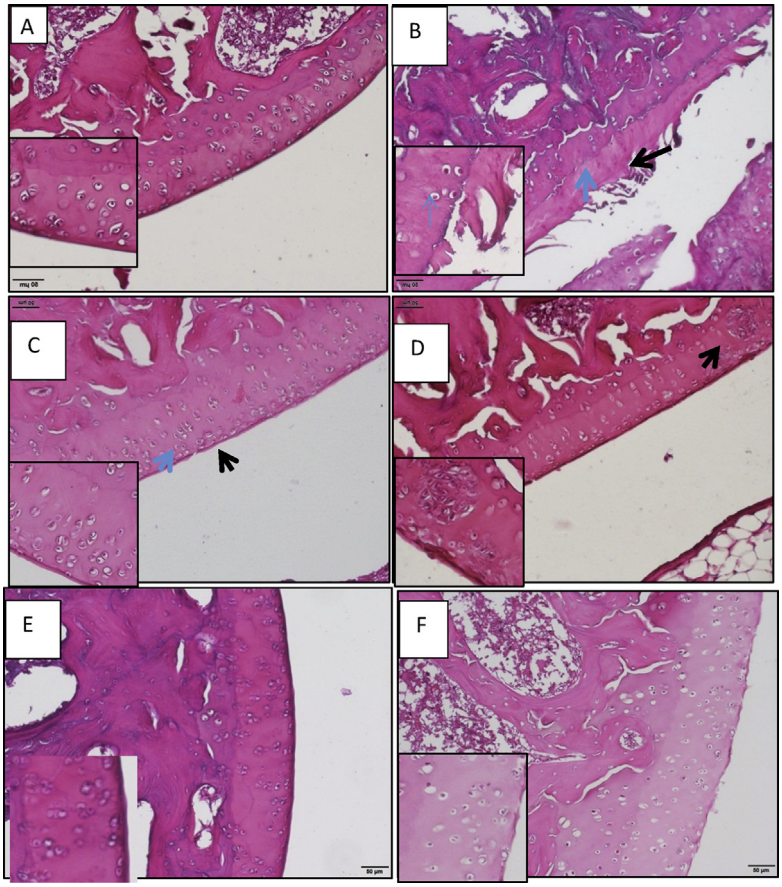

Figure 3. Histology of knee joint after stained by H&E.
A: normal; B: negative control; C: positive control; D: dose 1; E: dose 2; F: dose 3.

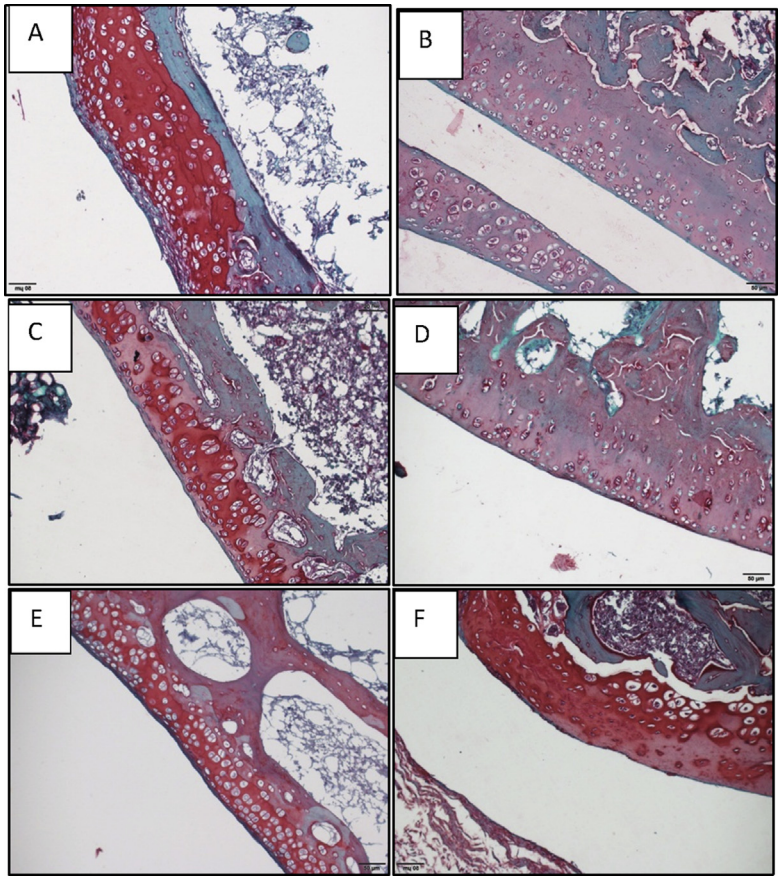

Figure 4. Histology of knee joint after stained by Safranin-O-fastgreen.
A: normal; B: negative control; C: positive control; D: dose 1; E: dose 2; F: dose 3.

in each group. The intensity of the red color of the normal control group looked more concentrated, while the negative control group had more faded red. The babandotan extract groups had a variety of color intensity. The red was a safranin-O binding to glycosaminoglycans in articular cartilage. The intensity of color using image J was evaluated as shown in Table 2.

3.5. Serum MMP-9 and TNF-α expression of OA rats

Table 2 showed that OA rats had higher level of TNF-α compared with control group ($P < 0.05$), therefore positive control and extracts treated rats showed lower level of TNF-α. Similar results also showed by MMP-9 results. These results suggested that babandotan extract could recover high amount of TNF-α to decrease the inflammation during OA and also recover high amount of MMP-9 to prevent cartilage degradation during OA.

4. Discussion

In this study, a rat model induced by monosodium iodoacetate. This model had extensively used as a laboratory model and has been reported successful to create OA rats model. Rats were induced by sodium iodoacetate in the left joint of rat intra-articular on day 1and then evaluated for OA four weeks after sodium iodoacetate stimulation, in order to formed OA rats model [13].

A. conyzoides leaves were purchased in Bogor, Indonesia, where local communities traditionally use babandotan leaves for the treatment of inflammatory disease in their daily live, such as fever, swelling, soreness, stiffness, wound, abdominal discomfort, *etc*. From preliminary screening, the result showed that the babandotan leaves extract was containing various secondary metabolites such as flavonoids, tannins, alkaloids, terpenoids-sterol, and saponins. TLC result showed flavonoids was equivalent to quercetin. Flavonoids have the core structure of the C6–C3–C6, two aromatic rings connected by 3 C atoms, typically with O atoms bond which form a heterocyclic oxygen bond. Quercetin, a member of flavonoids, have been extensively reviewed on its effects on inflammatory processes [14].

This study showed that the babandotan leaves extract can inhibit the process of OA significantly in sodium iodoacetate model rat (especially in dose 3: 160 mg/200 g BW) compared to the negative control group. Joint inflammation allowed the influx of inflammation cells and fluid into the inflamed area. The decrease of TNF-α concentration affects a decreased edema in the joint. The increased of edema of joint inhibition of the babandotan leaves extract showed that flavonoids had the anti-inflammation effect through the mechanism of action in inhibiting the activity of inflammatory cells and pro-inflammation cytokines in sodium iodoacetate -induced rat. The babandotan leaves extract dose used in the study was according to the previous studies and was explained that hydroalcoholic extract of *A. conyzoides* has low toxicity/LD50 (>5 000 mg/kg p.o.) which make it relatively safe to use [15].

The level of blood leukocytes test results showed that monosodium iodoacetate significantly induced inflammation and showed increasing in WBC number and lymphocyte than the normal group. Monosodium iodoacetate effectively increases the release of cytokines such as interleukin-1, interleukin-6, and TNF-alpha into the joint cavity and resulting enzymes to degrade the cartilage and damage the cartilage matrix [16,17]. It is shown that giving babandotan leaves extract can reduce the level of blood leukocytes compared to negative control, as well as the blood lymphocytes because the inflammatory process that occurred was inhibited by flavonoids iodoacetate sodium-induced rat caused chronic inflammation, which makes the level of blood leukocyte and lymphocyte increased on day 29. The babandotan leaves extract which contains flavonoids can inhibit the release of pro-inflammation cytokines such as TNF-α which can increase the infiltration of inflammatory cells into the inflamed area, that showed on day 50 [18]. This result indicated that babandotan extract could recover the changed in WBC and lymphocyte number. Extract of babandotan more effectively reduce the number of leukocytes compared with positive control, because it contained quercetin flavonoids which are known could reduce the migration of leukocytes consisting of monocytes, macrophages, lymphocytes and granulocytes to sites of inflammation by inhibiting the binding of leukocytes to the protein selectins that suppress the inflammatory process, as well as inhibit the migration of neutrophils [19].

The treatment by giving babandotan leaves extract which contained quercetin can decrease the concentration levels of TNF-α. Quercetin significantly inhibited TNF-α production and gene expression in a dose-dependent manner. Anti-inflammation effect of quercetin is produced by peripheral blood of mononuclear cells (PBMCs), which are mediated by inhibition of pro-inflammatory cytokine TNF-α via modulation of NF-κB. TNF-α is a pro-inflammatory cytokines which have an important role in the inflammatory process, such as increasing adhesion and infiltration of inflammatory cells into the inflamed area, increasing spending of other pro-inflammatory cytokines (such as IL-1, IL-2, IFN-γ), increasing the occurrence of angiogenesis, thus simplifying the pannus and causing damage to the articular cartilage and bone so that further aggravate the state of OA [20].

Histopathology was performed to evaluate curative effect of babandotan leaves extract on articular cartilage and the amount of proteoglycans. The smaller of the area and thickness of the articular cartilage, indicated that OA getting worse, and vice versa [21]. Table 2 was shown that the thinning of the articular cartilage in the negative control occurs because of the degradation of cartilage matrix. Induction of monosodium iodoacetate causing inhibition of the enzyme glyceraldehyde 3 phosphate on metabolic processes that lead to reduced ATP which leads to the death of chondrocytes [22,23]. Monosodium iodoacetate also activate proinflammatory cytokines such as IL-1 and TNF-α were triggered NO, COX-2 and PGE-2 in the inflammatory reaction. Activation of inflammatory mediators will cause chondrocytes to secrete enzymes such as matrix metalloproteinase causing cartilage degradation [24]. Chondrocyte death led to imbalance between anabolic and catabolic factors in cartilage matrix that caused the destruction and degradation of cartilage matrix.

In Safranin-O-fast green staining, safranin-O is a cationic dye staining of proteoglycans in the cartilage tissue. Safranin-O can bind to glycosaminoglycans and showed red. Fast green is a group containing sulfuric acid substrate to form strong bonds with amino groups on the protein and dye noncollagen part [25–27]. Safranin red intensity directly represents the amount of proteoglycans in cartilage tissue [28].

Flavonoids have pharmacological activity as anti-inflammatory effects. The mechanism of anti-inflammatory

flavonoid can be explained in some way such as inhibiting the activity of the enzyme cyclooxygenase (COX) and lipooxygenase (LOX), inhibition of leukocyte accumulation, inhibition of neutrophil degranulation, histamine release and inhibition anti-inflammatory activity (such as inhibiting production and activities pro-inflammatory cytokines). Quercetin, in particular, inhibits both COX and LOX, then diminishing the formation of these inflammatory metabolites [29].

Proteolytic enzymes, especially MMPs, are essential mediators of tissue destruction in both inflammatory and degenerative joint diseases [30]. There is ample evidence that MMPs, particularly the gelatinases MMP-2 and -9, are key enzymes in both inflammatory and degenerative joint diseases [31]. The results showed that quercetin treatment decreased the expressions of MMP-9. The level of pro-MMP-9 was found to be high in the 100 μM quercetin-treated cell lysate of PC-3 cells, suggesting inhibitory role of quercetin on pro-MMP-9 activation. Gelatin zymography study also showed the decreased activities of MMP-2 and MMP-9 in quercetin treated cells [32].

In conclusion, the study demonstrates a curative effect of quercetin-content babandotan leaves extract can prevent inflammation and proteoglycan degradation by inhibiting TNF-α and MMP-9 expression.

Conflict of interest statement

We declare that we have no conflict of interest.

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