

Anti-inflammatory and anti-hyperuricemia properties of chicken feet cartilage: treatment on gouty arthritis animal model

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ABSTRACT

Gout is a form of inflammatory arthritis caused by the deposition of uric acid. The therapeutic approach to gout is mainly divided by the treatment of inflammation and the management of serum urate level. This study aim to investigate whether chondroitin sulfate (CS) and glucosamine in chicken feet cartilage powder (CFE) and aqueous extract (AE) are able to decrease serum urate level and inflammation in animal model of gouty arthritis. CFE and AE were evaluated *in vitro* for xanthine oxidase (XO) inhibition. The anti-hyperuricemic activity and liver XO inhibition were evaluated *in vivo* on oxonate-induced hyperuricemia rats. Anti-inflammatory property was also determined on monosodium urate (MSU) crystal-induced paw edema model. CFE and AE supplementation showed urate-lowering activity. However, both treatments were not able to inhibit *in vitro* and *in vivo* XO activity. In MSU crystal-induced mice, the levels of paw swelling and lipid peroxidation were increased; in addition, a decrease in the activities of SOD and changes in the expression of CD11b⁺TNF- α and CD11b⁺IL-6 of the spleen were demonstrated. These changes were reverted to near normal levels upon CFE and AE treatments. These results suggest that CS and glucosamine from CFE and AE show a potent therapeutic effect against gouty arthritis.

INTRODUCTION

Gouty arthritis is a disease manifested as painful inflammation and occurs on the background of hyperuricemia or high plasma urate level ($> 408 \mu\text{mol/L}$) (Choi *et al.*, 2005; Fauci *et al.*, 2008; Orłowsky *et al.*, 2014). Hyperuricemia leads to urate crystal formation and deposition in the joints thus initiating inflammatory response by triggering proinflammatory mediator production, such as reactive oxygen species (ROS), tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6) (Ferrari *et al.*, 2016; Inokuchi *et al.*, 2008; Choi *et al.*, 2005). Medication prescribed to treat gout includes urate lowering agents and non-steroidal anti-inflammatory drugs (NSAIDs) but these agents are associated with intolerances or adverse effects (Busso and So, 2010; Khanna *et al.*, 2012; Liu-Bryan and Lioté, 2005; Umamaheswari *et al.*, 2009; Wang *et al.*, 2008; Wortman *et al.*, 2010). Thus, the development of novel natural agents with

therapeutic potential against gout would provide great clinical significance.

Chondroitin sulfate (CS) and glucosamine are two compounds present abundantly in the joint cartilage (de los Reyes *et al.*, 2000; Hoffman, 2001). The use of these compounds have become increasingly popular for treatment of arthritis as both are safe and have no adverse effect (Uebelhart *et al.*, 2004; Lovu *et al.*, 2008; Volpi, 2009; Nagaoka, 2014). In our previous research, CS and glucosamine had been successfully extracted from chicken feet cartilage using aqueous method. Chicken feet cartilage were incubated in boiling water for 150 minutes yielding aqueous extract (AE) which contained $2.04 \pm 0.15\%$ CS and $8.12 \pm 0.95\%$ glucosamine (Widyaningsih *et al.*, 2016). Previous research showed that CS significantly inhibited IL-1 β and TNF- α production from macrophages treated with MSU crystals (Orłowsky *et al.*, 2014). CS also affects the solubility of urate in joint fluids (Choi *et al.*, 2005). Nevertheless CS and glucosamine have never been tested in hyperuricemia. The aims of this study were to explore the anti hyperuricemic and anti-inflammatory properties of chicken feet cartilage aqueous powder (CFE) and extract (AE).

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MATERIALS AND METHODS

Materials and reagents

Chicken feet (broiler, 5-6 weeks old, 30 ± 5 g) were obtained from local market in Malang-Indonesia. All chemicals used were the highest analytic grade available obtained from Sigma-Aldrich (USA), Merck (USA), Megazyme (Ireland) and Nacalai (Japan).

Chicken feet cartilage powder preparation (Jalal *et al.*, 2012)

Chicken feet were washed thoroughly and boiled for 10 min. Cartilage was separated manually, grounded, and dried at 65°C for 24 h. Dried cartilage was grounded again to obtain chicken feet powder (CFE).

Chicken feet cartilage extract preparation (Shin *et al.*, 2006)

10 g of CFE was extracted with 100 ml of distilled water at 100°C for 2.5 h and then centrifuged (5000 rpm, 30 min). The supernatant was dried at 50°C and then grounded yielding chicken feet cartilage aqueous extract (AE). The glucosamine and chondroitin sulfate content were determined using the standard procedure previously described (Tsai *et al.*, 2012; Bitter and Muir, 1962).

In vitro xanthine oxidase assay (Ferrari *et al.*, 2016)

CFE, AE and allopurinol (as positive control) were solubilized in DMSO (1%) and distilled water in order to obtain concentrations 10; 20; 30; 40; 50 and 100 $\mu\text{g}/\text{mL}$. 500 μL of each sample was added into 1.125 mL of phosphate buffer (pH 7.4) and 187.5 μL of XO enzyme (0.28U/mL). This solution was incubated for 10 min at 30°C . Then, reaction of enzyme began after adding 1.375 mL of xanthine substrate. The absorbances were obtained at 295 nm (Shimadzu) every minute for 10 min. The results were calculated using formulation: $(1 - \text{test inclination}/\text{blank inclination}) \times 100$ and expressed as percentage of XO inhibition.

Experimental animals (rats and mice)

Specific pathogen-free male BALB/c mice (25-30 g) and male wistar rats (150-180 g) were used in this experimental. The experimental procedures in this study were approved by Brawijaya Ethical Committee under registration number 563-KEP-UB and carried out in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institute of Health (NIH Publication, revised in 1985). All animals were purchased from *Malang Murine Farm* (Malang, Indonesia) and divided into experimental groups ($n=5$). Animals were housed in individual plastic cages with controlled environment. Water and standard chow were given *ad libitum*.

Preparation of drugs and test solutions for *in vivo* methods

Potassium oxonate suspension, CFE solution, AE solution and allopurinol solution were given based on body weight of each animal. CFE and AE were dissolved in distilled water and drugs (allopurinol and indomethacin) were solubilized in Na-CMC 0.8% (vehicle). Potassium oxonate and monosodium urate were

suspended in sterile saline 0.9% before its use.

Anti-hyperuricemic and inhibition of liver XOD activity (Ferrari *et al.*, 2016)

In order to evaluate the anti-hyperuricemic and inhibition of liver XOD activity of CFE and AE, animals were divided into five groups ($n=5$) and injected with potassium oxonate to the animals of groups 2-5 (300 mg/kg I.P.) on the first and third day of the experiment (Watanabe *et al.*, 2006; Lima *et al.*, 2015). Once a day, 1 h after the potassium oxonate injection, treatments were given by oral gavage. Non-hyperuricemia group (group 1) and hyperuricemia group (group 2) were administered with vehicle. Group 3 was treated with allopurinol (10mg/kg P.O.). Groups 4 and 5 were treated with CFE dose 208 mg/kg and AE dose 333 mg/kg. Those doses were chosen based on daily recommended intake of glucosamine (1500 mg) and chondroitin sulfate (1200 mg) in human (Hyde, 2005). On the third day, 1 h after treatments, rats were anesthetized with ketamine dose 100 mg/kg and xylazine dose 20 mg/kg. Rats blood were obtained from abdominal aorta and centrifuged at 1500 g for 5 min. Supernatant were collected and centrifuged at 3000 g for 10 min until complete sera separation. These materials were used for uric acid quantification. Rats livers were also collected and washed in cold saline solution (0.9%) for XOD activity determination

MSU crystal-induced inflammation in mice (Rasool and Varalakshmi, 2006)

MSU crystal was prepared according to procedure previously described (Saegmiller *et al.*, 1962). Inflammations were induced by MSU-crystal injection in subplantar region of the mouse right hind paw. All groups, except negative control group, received 0.1 ml (40 mg/ml) MSU crystal injection. CFE (30.04 mg/20 g), AE (48.09 mg/20 g), and indomethacin (0.39 mg/20 g) were given orally by gavage 1 h prior to injection and repeated daily for the next 2 days. Edema formation was determined by measuring paw thickness at 0, 4, 24, and 48 h after MSU crystal injection using a caliper rule. Mice were sacrificed at the end of study. Blood was collected from the inferior vena cava for MDA and SOD assay, while spleen was quickly removed and rinsed with cold saline for CD11b⁺TNF- α and CD11b⁺IL-6 assay using Flowcytometer.

Statistical analysis

The results are expressed as mean \pm SD, and significant differences between the variables were analyzed using Minitab 16. Results were defined as statistically significant at $P < 0.05$.

RESULTS

In vitro xanthine oxidase inhibitory activity

Allopurinol, a xanthine oxidase inhibitor, was used as positive control and showed an inhibition of 89.75% (100 mg/mL) and IC_{50} of 50.01 mg/mL. However, as shown in Table 1, chicken feet cartilage powder (CFE) and aqueous extract (AE) did not show any inhibition against xanthine oxidase.

Table 1: *In vitro* inhibitory xanthine oxidase activity of extract from chicken feet cartilage.

Sample	Concentration (µg/ml)	Inhibition (%)	IC ₅₀ (µg/ml ± S.D)
Allopurinol	100	89.75	50.01 ± 2.76
CFE	100	-	-
AE	100	-	-

Chicken feet cartilage powder (CFE), aqueous extract from chicken feet cartilage (AE). Data present mean S.D (n=5).

Effects of CFE and AE on serum urate levels in hyperuricemic rats

Mean of serum urate levels in different experimental groups were illustrated in Figure 1. The intra-peritoneal injection of potassium oxonate solution was allowed significant increase (P<0.05) in serum urate levels in hyperuricemia group compared to normal control group. CFE and AE treatment were able to significantly reduce serum urate levels compared to hyperuricemia group. Allopurinol also promoted a significant (P<0.05) reduction on serum urate levels of hyperuricemic rats to value lower than in hyperuricemic animals.

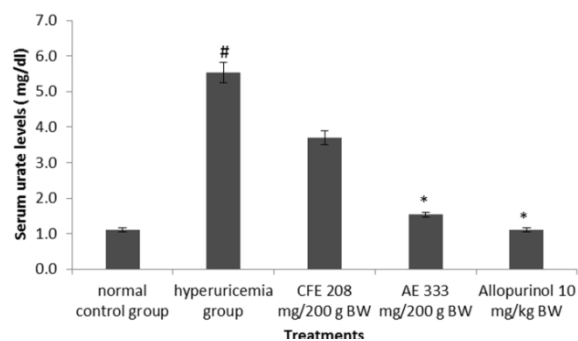


Fig. 1: Anti-hyperuricemic effects of chicken feet cartilage powder (CFE) and aqueous extract from chicken feet cartilage (AE) in rats pretreated with potassium oxonate. Experiments were performed as described in Materials and Methods. Data present mean S.D (n=5). One- way ANOVA followed by post hoc test (Least Significance Different) used for statistical significance. *P<0.05 compared with hyperuricemic control group; #P<0.05 compared to normal control group

Effects of CFE and AE on xanthine oxidase activity in rat liver

The inhibitory activity of CFE and AE against xanthine oxidase was shown in Table 2. There was no xanthine oxidase inhibition observed in hyperuricemia group, as xanthine oxidase do not inhibited by potassium oxonate. In addition, treatment with CFE and AE also did not significantly (P>0.05) inhibited liver xanthine oxidase activity whereas allopurinol inhibited XOD activity by 49.90%.

Table 2: Effects of CFE and AE on xanthine oxidase activity in rat liver *in vivo*.

Treatments	XOD activity (U/mg protein)
Normal control group	1.51±0.87
Hyperuricemia animals	1.06±0.44
CFE	1.25±0.51
AE	0.61±0.41
Allopurinol	0.33±0.19*

Chicken feet cartilage powder (CFE), aqueous extract from chicken feet cartilage (AE). Data present mean S.D (n=5). One- way ANOVA followed by post hoc test (Least Significance Different) used for statistical significance. *P<0.05 compared with normal control group

Edema formation and inhibition in mice

To established the MSU-crystal inflammation, time course of edema formation in mice was conducted. MSU-crystals induced acuted inflammation as compared with the control group. Edema began to develop at first 4 h and reach its peak in 24 h (Figure 2). On the other hand, both CFE and AE groups considerably showed reduction (P<0.05) in the inflammation. Inhibition in positive control was used as a comparison to determine inhibitory activity of AE and CFE treatment (Table 3). Inhibition rate of edema was calculated as follows: % edema inhibition = $(V_{24} - V_{48}) \times 100 / V_{24}$, where V₂₄ and V₄₈ were paw thickness at 24 h and 48 h, respectively. Based on the statistic analysis, AE performed a significant (P<0.05) reduction in inflammation while CFE performed a reduction but not statistically significant (P>0.05).

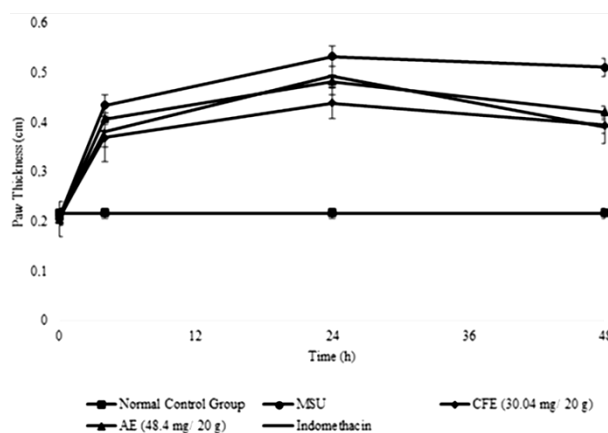


Fig. 2: Effects of chicken feet cartilage powder (CFE) and aqueous extract from chicken feet cartilage (AE) on paw edema in mice. Experiments were performed as described in Materials and Methods.

Table 3: Edema inhibition of CFE and AE on paw edema in mice.

Group	Edema Inhibition (%)
Normal Control Group	-
MSU	4.13 ± 0.75
CFE	10.12 ± 4.29
AE	16.01 ± 9.42*
Indomethacin	20.40 ± 6.35*

Chicken feet cartilage powder (CFE) and aqueous extract of chicken feet cartilage (AE). Values were expressed as mean±SD of 5 animals. One- way ANOVA followed by post hoc test (Least Significance Different) used for statistical significance. *P<0.05 compared with MSU crystals injection groups.

Lipid peroxidation and anti-oxidant status in mice

MSU crystals injection triggered the oxidative stress in positive control group that was measurable in terms of increased MDA level and reduced SOD activity. Supplementation with AE and CFE had resulting in lower MDA level compared with positive control group although the difference was not significant (P>0.05). In addition, AE and CFE administration showed a significant improvement (P<0.05) in SOD activity similar to that indomethacin group when compared with positive control group.

Table 4: MDA level and SOD activity in blood serum.

Group	MDA level (ng/ml)	SOD activity (U/ml)
Negative control group	695 ± 87.74	3.34 ± 0.34 [*]
MSU	779 ± 102.39	2.26 ± 0.67
CFE	752 ± 37.35	2.84 ± 0.48 [*]
AE	727 ± 93.10	3.19 ± 0.28 [*]
Indomethacin	701 ± 58.61	3.22 ± 0.60 [*]

Chicken feet cartilage powder (CFE) and aqueous extract of chicken feet cartilage (AE). Values were expressed as mean ± SD (n=5). One- way ANOVA followed by post hoc test (Least Significance Different) used for statistical significance. *P<0.05 compared with MSU crystals injection group.

Effects on pro-inflammatory cytokines in mice

To examine the effect of CFE and AE on the production of inflammatory cytokines induced by MSU, TNF- α and IL-6 expression by macrophage were determined in spleen. As represented in Table 5, under basal condition, macrophages produced low pro-inflammatory cytokines (CD11b⁺TNF- α and CD11b⁺IL-6) and MSU-crystal stimulation significantly increased the expression. However, CFE and AE were able to decreased pro-inflammatory cytokines expression in spleen (P<0.05) compared to the positive control group.

Table 5. Relative percentage of CD11b+TNF- α dan CD11b+IL-6

Group	% TNF- α	% IL-6
Negative control group	8.96 ± 2.72 [*]	20.34 ± 11.29 [*]
MSU	24.32 ± 9.17 [#]	43.82 ± 9.75
CFE	19.41 ± 6.52 [#]	26.02 ± 8.80 [*]
AE	16.61 ± 4.93	25.29 ± 4.33 [*]
Indomethacin	22.33 ± 8.66 [#]	20.99 ± 6.36 [*]

Chicken feet cartilage powder (CFE) and aqueous extract of chicken feet cartilage (AE). Values were expressed as mean ± SD of 5 animals. One- way ANOVA followed by post hoc test (Least Significance Different) used for statistical significance. *P <0.05 compared with MSU crystals injection group; #P <0.05 compared to negative control group.

DISCUSSION

Gout is a type of arthritis whose pathogenesis generally associated with hyperuricemia or high levels of uric acid (>6.8 mg/dL) (Fauci *et al.*, 2008; Neogi, 2011). It usually comes with recurrent inflammation, intense pain, and red-swollen joint. As the prolonged use of synthetic chemical medicine associated with many side effects, a novel natural agent against gout was developed from glucosamine and CS. A number of studies have reported that glucosamine and CS possess anti arthritic action via various pathways (Chan *et al.*, 2005). In our previous study, both glucosamine and CS had been successfully extracted from chicken feet cartilage and possess anti-inflammatory effect against carrageenan-induced paw edema model in rats. In the present study, glucosamine and CS in the form of CFE and AE were used to treat animal model of gouty arthritis.

In the present study, treatment of CFE and AE were not capable to inhibit xanthine oxidase activity both *in vitro* and *in vivo*. Based on this result, it can be concluded that CS and glucosamine were able to lower serum urate level through the other mechanism. Administration of CFE on rats was not significantly reduced (P>0.05) serum urate levels due to the big molecular weight of CS polymer in CFE resulting in lower bioavailability compared to AE (Adebawale *et al.*, 2000).

Reduction of serum urate levels in AE group may be due to the chondroitin sulphate and glucosamine are able to dissolve uric acid in rats. The solubility of uric acid may be due to chondroitin sulfate and glucosamine have the ability to draw water. Chondroitin sulfate and glucosamine are compound which contain negative charge in sulfate and carboxylic cluster, so that negative charge will attract water molecules because it contains positive ions H⁺ (Prydz and Dallen, 2000). Furthermore, the more water bound up with chondroitin sulfate and glucosamine, fluid volume in rats will reduce to a decline in blood pressure. Falls in blood pressure activity in rats cause the activation of system renin angiotensin aldosterone system (RAAS). A mechanism of chondroitin sulphate and glucosamine in lowering the levels of uric acid different to the allopurinol mechanism. Allopurinol capable to inhibit the activity of xanthine oxidase through the mechanism acts as a compound similar to the substrate enzyme xanthine oxidase or purine binding site (Lima, 2015).

Besides hyperuricemia, inflammation also reported as a major problem that attacks gout patients. After MSU-crystal injection, paw thickness was significantly increased (P<0.05) compared to normal group. This increase in paw thickness probably caused by increase in vascular permeability resulting in edema formation. CFE and AE administration was able to inhibit the edema formation. Inhibition in edema formation was began to be seen at first 4 hour and remained in subsequent hours. This inhibition effect observed might be associated with anti inflammatory activity of glucosamine and CS present in CFE and AE (Du Souich *et al.*, 2009).

In the patients with gout, there is a production of free radical caused by phagocytosis of MSU crystal. Production of free radical would ultimately lead to lipid peroxidation and impair anti-oxidant status (Murunikkara and Rasool, 2014). Those change can be measured as decreasing SOD activity and increasing in MDA level (Bottegoni *et al.*, 2014). Administration of AE and CFE caused a reduction in MDA level and improvement in SOD activity. The mechanism seems to involve antioxidant properties of CS and glucosamine. The previous study had revealed that CS can act as antioxidant by increasing endogen antioxidant that can be measurable by SOD activity (Egea *et al.*, 2010). Glucosamine supplementation also mediate anti-inflammatory effects by acting as antioxidant and decreasing nitric oxide synthase expression and activity, and consequently the oxidative stress generated by ROS (Valvason, Musacchio, & Pozzuoli, 2008). Our result demonstrated that CFE and AE also can reduced TNF- α and IL-6 expression in spleen. Indomethacin, a non-steroidal anti inflammatory drugs (NSAID) often prescribed for gout patients, also reduced CD11b⁺TNF- α dan CD11b⁺IL-6 level in spleen. Those cytokines were produced by various cells, including synovial cells, monocytes-macrophages, and neutrophils, and were well known to propagate a local or systemic inflammatory response (Liu-Bryan and Lioté, 2005; Inokuchi *et al.*, 2008; Schiltz *et al.*, 2002). It can be stated that AE and CFE inhibits pro-inflammatory cytokines via NF κ B-dependent mechanism. NF κ B is closely associated with pro-inflammatory cytokine production,

such as IL-6 and TNF- α . Since NF κ B is a key player in inflammatory response, inhibition of it can be beneficial for modulating inflammatory diseases (Krishnan *et al.*, 2014). The potent inhibitor of TNF- α and IL-6 by glucosamine is due to inhibition of NF κ B and production of PGE₂. In addition, CS also acts on the nuclear translocation of NF κ B (Bottegoni *et al.*, 2014).

CONCLUSION

The present study demonstrated that glucosamine and CS from AE and CFE were remarkably able to ameliorate the gouty arthritis by decreasing serum urate level but not able to inhibit xanthine oxidase activity. In addition, the AE and CFE also possess anti-inflammatory activity through inhibition of pro-inflammatory mediator such as ROS, TNF- α and IL-6. Thus this study provides insights on possible utilization of CFE and AE as an anti-gouty arthritic-supplement. These evidences also gives information regarding their anti-hyperuricemic and anti-inflammatory mechanism against gouty arthritic. However further investigations, will be necessary to investigate whether CFE and AE produces a similar therapeutic efficacy in human as was observed in this study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Conflict of Interests: There are no conflicts of interest.

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