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Acute Toxicity and Suppressive Effects of a Curcumin Analogue Gamavuton-0 (Gvt-0) On CFA-Induced Arthritis in rats

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ABSTRACT

Gamavuton-0 (GVT-0) is a curcumin analogue, which is synthesized from acetone and vanillin with chloride acid as a catalyst and ethanol as a solvent. The compound has been reported to have an anti-inflammatory effect related to its activity as COX-2 inhibitor, anti-oxydant, and radical scavanger. This study was aimed to investigate whether the GVT-0 has a suppressive effect on rheumatoid arthritis (RA) as one of chronic inflammatory disorders in a rat model. Wistar rats were immunized with Complete Freund's Adjuvant (CFA). After a second CFA immunization, the rats were treated with GVT-0 orally at 10, 20, 40, 80 mg/kg BW once a day for 21 days, while the positive control received methotrexate 0.22 mg/kg BW. The animal paws were evaluated macroscopically for redness, swelling and deformities with Smit method to assess arthritic index. The anti-inflammatory effect of GVT-0 was evaluated using a plethismograph by measuring rat paw edema, while it's effects on the level of TNF-α and IL-1β in the ankle joints were examined using an ELISA method. The effect of GVT-0 on cartilage destruction was assesed histologically using Safranin-O staining. The acute toxicity test was also performed to assess the safety potential of the compound. The oral treatment of rats for 21 days with various doses of GVT-0 significantly suppressed the progession of RA indicated by the improvement of arthritic index and decreased the inflammation in rat paws. The compound also decreased the level of TNFα and IL-1β in ankle joints. The destruction of cartilage was significantly reduced in rats ankles after treatment with GVT-0. In toxicological assay, the apparent LD_{50} value of GT-0 was regarded as 7,29 g/kg BW and was classiefied to be practically non-toxic. The results suggest that GVT-0 is safe and potential to modify the progression of rheumatoid arthritis and can be developed as a new disease modifying anti rheumatoid arthritis drugs (DMARDs).

INTRODUCTION

Gamavuton-0 (GVT-0), with an IUPHAC name of 1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadiene-3-one, is an analogue of curcumin, which is synthesized from vanillin and acetone via condensation reaction of Claisen-Schmidt (Sardjiman, 2000). It is resulted by modifying the middle site of the chemical structure of 1,7-diphenyl-1,6-heptadien-3,5-dion in curcumin to be 1,5-diphenyl-1,4-pentadien-3-on in GVT-0. It, therefore, differs from curcumin in the bridge between the two aromatic rings. GVT-0 has a shorter chain with one carbonyl and no methylene group (Figure 1). Removal of the methylene and carbonyl group leads this new compound to become more stable than curcumin without affecting the antioxidant effect (Sarjiman, 2000). It has been already patented in The United States (Patent no. US 6.777.447 B2, 2004) for the synthesis. The optimal mole composition of acetone: vanilin for GVT-0 synthesis is 1:2, which produced 11.43 % yield of GVT-0. GVT-0 has been reported to have an anti-inflammatory effect related to its activity as a COX-2 inhibitor (Mayasari, 2008), anti-oxydant, radical scavenger (Yuniarti, 2006), and antibacterial activity (Sardjiman, 2000). GVT-0 was also reported to have analgesic effects, both in acute and persistent pain (Ikawati, et al, 2014). Rheumatoid arthritis (RA) is characterized by chronic inflammation of the synovium, progressive erosion of the articular cartilage through pannus formation, and joint destruction (Zhao *et al.*, 2006).

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The main pathology of the affected synovial tissue consists of hyperplasia and subintimal infiltration of T and B lymphocytes. Synovial tissue hyperplasia forms the pannus tissue that irreversibly destroys the cartilage and bone in the affected joint. RA progression is associated with elevated levels of tumour necrosis factor-α (TNF-α) and interleukin (IL)-1β produced by macrophages and dendritic cells, an imbalance of Th1/Th2 and overproduction of antigenspecific immunoglobulins (Panayi, 1997). Most of the current treatments are directed to correction of the immune aberration that supposedly drives synovial cell proliferation and cartilage erosion. In the present research, we study the potential effect of GVT-0 in supressing the progression of RA, in order to discover a new candidate for anti RA.

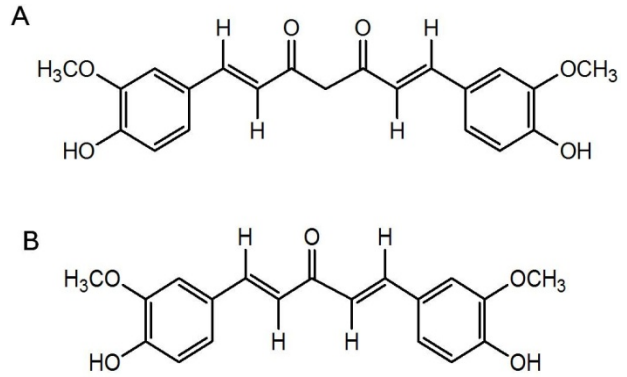

Fig. 1: Comparison between the structure of curcumin (A) and Gamavuton-0 (1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadiene-3-one (B).

METHODS

Pharmacological Assay

Materials

GVT-0 was obtained from Curcumin Research Center Faculty of Pharmacy Gadjah Mada. The compound was tested for its purity using a thin layer chromatography (TLC) and gas chromatography (GC). To confirm the compound is GVT-0, its structure was elucidated using a mass spectrophotometry, infrared spectrometer, and proton nuclear magnetic resonance (H^1-NMR) spectrometer.

Animals

Female Wistar rats weighing 120-200 g were obtained from the Animal Research Development Unit, Faculty of Pharmacy, Gadjah Mada University. The rats were maintained under standard housing conditions in wire-bottom, plastic cages and provided with food and water ad libitum. They were kept in a 12:12-h light-dark cycle in a temperature-controlled room (27±2oC). At the start of the experiments, the rats were 6-8 weeks old.

Induction of CFA and GVT-0 treatment

The ethical clearance for conducting in vivo experimental analysis in animals has been obtained from Ethical Committee for Health Research Gadjah Mada University, Yogyakarta. The rats were induced by subplantar injection of 100µl Complete Freund's Adjuvant contained 1 mg/ml *Mycobacterium tuberculosis* (Sigma Aldrich, Germany) in the right hindpaws. On day 21, all the animals were boosted with a subplantar injection of 50 µl CFA. The next day, twenty six rats were selected randomly and divided into six groups, which each comprised of four rats.

The control negative group were treated orally with 0.5% Na-CMC, the control positive group were treated orally with methotrexate 0.22 mg/kg BW, the GVT-0 treated group were received the GVT-0 administered orally with the doses of 10, 20, 40 and 80 mg/kg BW, respectively, for 21 days. The gradual onset of arthritis normally starts approximately 3 weeks after the initial immunization. The progression of arthritis was evaluated by macroscopic scoring of the paws every 2 days and histological analysis of the ankles on day 42.

Macroscopic scoring of arthritic index and measurement of paw edema volume

The clinical severity of arthritis was evaluated from the arthritic index dan paw edema volume that was graded every other day, based on the changes in swelling and redness of toes, foot pad, and ankle, with a maximum score of 2 per paw for arthritic index according to Smit method (Smit *et al.*, 2000). The macroscopic symptoms scores were as follows: swelling and redness of 1 toe = 0.25, swelling and redness of at least 2 toes = 0.50, swelling of foot pad = 0.75, swelling and redness of toes and swollen foot pad =1.00, swelling and redness of toes and foot pad = 1.25, swelling and redness of toes and minor swelling of foot pad and ankle = 1.50, swelling and redness of toes and major swelling of foot pad and ankle = 1.75, and swelling and redness of toes, foot pad, and ankle = 2.00. Two persons independently performed the scoring, and the mean cumulative value for all paws was taken as the arthritic index (AI). The animals were considered to have arthritis when AI was at least 1. Paw edema volume was measured using a plethismograph.

Histological Analysis

On the day 42, the rat angkles were removed after euthanasia with ether and stored in 10% formalin (Asia Lab.) for 24 hours. Decalcification in $AlCl_3$, 37% HCl, 58% formic acid, liquid paraffine, and distilled water for 5 days was subsequently carried out and the preparations were embedded in paraffine. After the removal of the paraffine, 3-µm thick sections were cut. Safranin-O staining was performed to estimate the proteoglycan content in the cartilage (Greaves, 2000).

The preparations were analyzed under defined conditions using a light microscope Ken-a-Vision® T-1952-230 (40 × magnification), and the results were stored in a software Soft Applied Vision. All slides were evaluated by an independent observer who was blinded to the design and details of the study based on a modified Roth's method (Roth *et al.*, 2005). In all cases, three sections per ankle joint were examined and scored

using a semiquantitative scale for each 2 slices. Score 1 for minimum intensity, score 2 for middle intensity, and score 3 for maximum intensity (Figure 2).

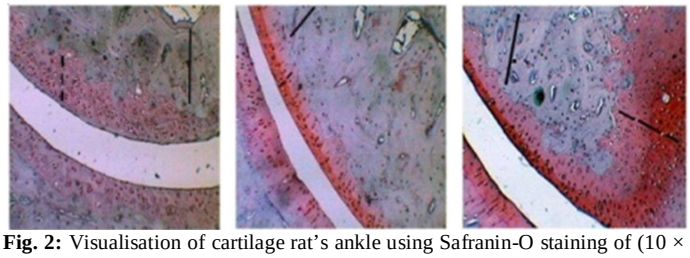

Fig. 2: Visualisation of cartilage rat's ankle using Safranin-O staining of (10 × magnification). Cartilage destruction is indicated by diminished color intensity. (a) Score 1 for minimum intensity, (b) score 2 for middle intensity, and (c) score 3 for maximum intensity.

Measurement of cytokine levels in ankle joints

TNF-α and IL-1β levels were measured using commercially available ELISAs assay (BioSource International, Inc., California, USA) according to the manufacturer's recommendations. For measuring the cytokine production, peeled joint tissues from the upper portion of ankle to the middle of the paw were ground by homogenizer in the equal volume of the lysis buffer (100 mmol/l potassium phosphate, pH 7.8). The tissue lysates were used to measure the level of cytokines. Cytokine production was standardized as the amount of cytokine per ml of lysates.

Statistical analysis

Statistical evaluation was carried out using the SPSS with One Way ANOVA test then Post Hoc Test LSD method. Data were expressed as means and standard errors of the means. $P \leq 0.05$ was considered statistically significant for

Toxicological assay

Animals

Female Wistar rats with body weights ranging from 150 to 180 g were used in this study. The animals were maintained in the same environmental conditions of temperature controlled room (27 ± 1^{o}C), and illumination (12x12 h light-darkness cycle) and maintained with free access to water and food**.**

Acute toxicity studies

Thirty two female Wistar rats were acclimatized for a week in cleaned cages. The rats were randomly divided into 4 groups (one control group and three experimental groups) with each group comprised 8 rats. Three experimental groups were orally administered with GVT-0 suspension in 0.5% Na-CMC solution with the dose of 10, 270 and 7.290 mg/kg BW, respectively. The control group received only the vehicle (0.5% Na-CMC solution), at a volume of 5 ml/100 g BW . The animals were observed every hour from the day of treatment. The nature and time of any adverse effect occurred were documented. Observation was carried out for 14 days. On the day 14, all animals were weighed, sacrificed and gross pathologic examination was then conducted. Sections of tissues such as lung, kidney, spleen, stomach, liver, and heart were obtained for histopathological studies.

Determination of median lethal dose (LD50)

The death of animal was monitored over a period of 24 h. The acute toxicity LD50 was calculated as the geometric mean of the dose resulted in mortality and that which caused no mortality at all. The animals were observed and the studies terminated after two weeks. Recovery and body weight gain after each investigation were taken as a sign of surviving the test.

Histopathological studies

All the sacrificed rats were necropsied. Specimens were collected from various organs and fixed in 10% neutral formalin buffer. Paraffin sections (6-8 micron) were prepared and stained with Haematoxylin and eosin for microscopic examination

RESULTS

Effect of GVT-0 on inflammation and arthritic index

On day 2, arthritis developed as a significant swelling of the right ankle joint in all animals. The administration of GVT-0 for 21 days with the dose of 10, 20, 40, and 80 mg/kg BW could decrease paw edema volume. The comparison of anti inflammatory activity between GVT-0 in various dose and methotrexate (MTX) as the postive control is shown in table 1.

As shown in table 1, there was a tendency of dose-dependent activity of anti inflammatory action, with the dose of 80 mg/kg BW of GVT-0 giving the maximal suppression effect on rat paw edema. However, there was no significant difference statistically ($P > 0.05$).

Table 1: Anti inflammatory activity of MTX and GVT-0 in various doses on CFA-induced arthritis in rats.

Group	Anti inflammatory activity (% ± SEM)
MTX 0.233 mg/kgBW	46.68 ± 6.21
GVT-0 10 mg/kg BW	40.96 ± 8.72
GVT-0 20 mg/kg BW	44.25 ± 7.34
GVT-0 40 mg/kg BW	46.42 ± 8.29
GVT-0 80 mg/kg BW	50.04 ± 7.63

Values are expressed as Mean ± SEM, p<0.05 when compared with control (One-way ANOVA)

Table 2: Suppressive effect of MTX and GVT-0 on arthritic index in various doses on CFA-induced arthritis in rats.

Group	Supressive effect on arthritic index (% ± SEM)
MTX 0.233 mg/kgBW	17.81 ± 2.01
GVT-0 10 mg/kg BW	15.15 ± 3.22
GVT-0 20 mg/kg BW	17.97 ± 2.45
GVT-0 40 mg/kg BW	19.74 ± 3.27
GVT-0 80 mg/kg BW	20.06 ± 3.51

Values are expressed as Mean ± SEM, p<0.05 when compared with control (One-way ANOVA)

While in the terms of arthritic index, various doses the GVT-0 also showed supressive activity, as demonstrated in table 2. Again, the dose of 80 mg/kg BW tends to give the highest effect, despite

no statistical differences with the smaller doses. However, the progression of arthritis was significantly delayed in rats treated with GVT-0 compared to the negative control rats, with suppressive effect of 20.06% at dose 80 mg/kg.

Inhibition of cartilage destruction

Cartilage destruction was scored based on its intensity of Safranin-O staining (Figure 2). The maximum intensity of safranin-O staining was found in normal group (without induction of CFA) indicating the cartilage remained intact, while the lowest intensity was found in the negative control group (CFA-induced and received only 0.5% Na-CMC). The administration of various doses of GVT-0 could inhibit cartilage destruction, as demonstrated by Safranin-O staining assay (Figure 3), with the score displayed in table 3.

Table 3: Protective effect on cartilage destruction of MTX and GVT-0 in various doses.

Group	Protective effect of MTX and GVT-0 on cartilage destruction (% ± SEM)
MTX 0.233 mg/kgBW	71,43 ± 6.75
GVT-0 10 mg/kg BW	14,29 ± 3.12
GVT-0 20 mg/kg BW	20,21 ± 2.78
GVT-0 40 mg/kg BW	29.45 ± 3.73
GVT-0 80 mg/kg BW	57,14 ± 4.32

Values are expressed as Mean ± SEM, p<0.05 when compared with control (One-way ANOVA)

The potential inhibition of cartilage destruction was calculated based on scoring of Safranin staining. The protection effect of GVT-0 and MTX is displayed in table 3. MTX showed the highest protection effect on cartilage destruction, while GVT-0 demonstrated dose-dependent effects. The GVT-0 with the dose 80 mg/kg BW showed the highest protection effect on cartilage destruction, compared to the other doses.

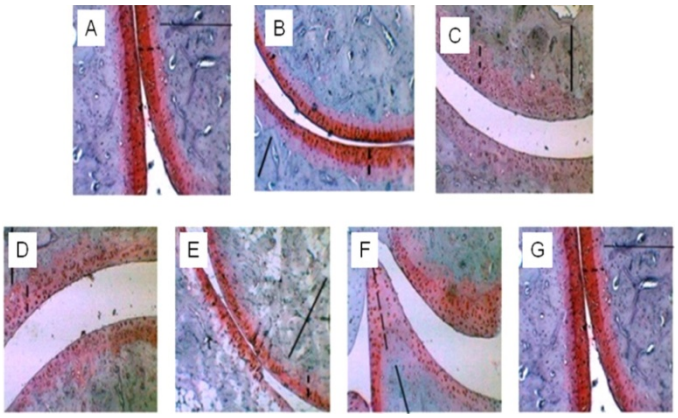

Fig. 3: Visualisation of cartilage rat's ankle using Safranin-O staining of (10 × magnification). A) normal; B) MTX; C - F) GVT-0 10, 20, 40, and 80 mg/kg BW respectively, G) 0.5% Na-CMC.

Effects of GVT-0 on levels of TNF-α and IL-1β in the rats ankles

As the citokines mostly contribute to development of RA, TNF-α and IL-1β in the rats ankles were measured. The levels of these surrogetes in control and treatment groups are displayed at Figure 4 and 5. As shown in the figure, the level of TNF-α and IL-1β were all significantly reduced by the treatment with MTX and GVT-0 compared to control rats treated with the vehicle (0.5% Na-CMC, $P< 0.05$). These results suggest that GVT-0 inhibits the production of proinflammatory cytokines in the affected paws of RA rats, where 40 mg/kg BW was optimum dose of GVT-0 in decreasing TNF-α levels at rats ankles. The GVT-0 could decrease TNF-α levels with an oral ED_{50} value of 65.22 mg/kg BW per oral. In the case of IL-1β, the dose of 80 mg/kg BW gave the highest suppression effect on IL-1β compared to the other doses of GVT-0, or MTX dose of 0.223 mg/kg BW. GVT-0 could decrease IL-1β levels with an oral ED_{50} of 12.38 mg/kg BW.

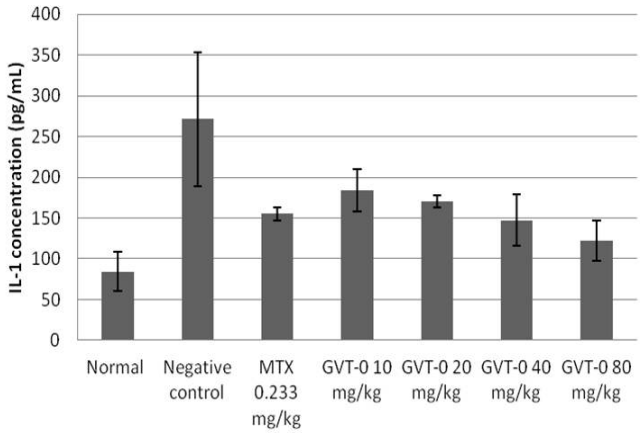

Fig. 4: The level of TNF-α in the rats ankle tissue, either in normal, control, and treatment with GVT-0 or MTX. Ankle joint tissues were prepared from joint tissues and analysed for TNF-α using an ELISA method. Data are mean ± S.E.M. of 4 joints.

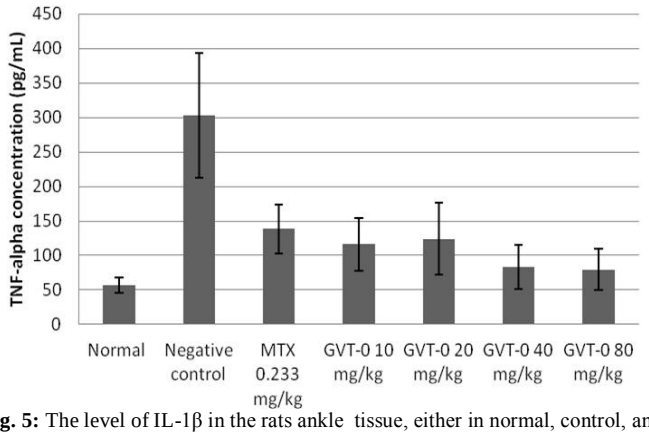

Fig. 5: The level of IL-1β in the rats ankle tissue, either in normal, control, and treatment with GVT-0 or MTX. Ankle joint tissues were prepared from joint tissues and analyzed for IL-1β using ELISA method. Data are mean ± S.E.M. of 4 joints.

Toxicological assay

There was no toxic symptoms occurred during the acute toxicity test after the administration of GVT-0. The compound also had no effect on the vital organs, both macroscopic or microscopically. Moreover, there was no animal death until the end of the test, with the dose of up to 7.29 g/kg BW. Therefore, the apparent LD_{50} value of GT-0 was regarded as 7,29 g/kg BW and was classified to be practically non-toxic (Loomis, 1978).

DISCUSSION

GVT-0 has been found to inhibit inflammation (paw edema volume) and to reduce the arthritic index, and also to protect cartilage destruction in the rats. These effect are probably due to the mechanism of GVT-0 as radical scavenger such as OH• that is largely produced in the inflammation site by neutrophil activation.

The presence of –OH phenolic group in GVT-0 is responsible for its activity as a radical scavenger (Yuniarti, 2006), whereas the contribution of $–OCH_3$ group as an electron donating group may increase electron density of π (phi) binding. Therefore, it can increase the activity in radical scavenging. The increase of radical scavenging activity may correspond to the inhibition of cyclooxygenase-2 enzyme (COX-2) that converts arachidonic acid into prostaglandine (Mayasari, 2008). It is therefore GVT-0 is able to inhibits CFA-induced inflammation and arthritis in rats via the free radical scavenging activity.

IL-1β and TNF-α are structurally unrelated cytokines that bind to different cellular receptors. Yet their spectra of biologic effects overlapped include their ability to enhance the activation of helper T (T_H) lymphocytes by antigen-presenting cells (APCs) and also their ability to increase collagenase and bone resorption by osteoclasts (Stites *et al.*, 1997). These cytokines have been detected in the synovial (joint) fluid of RA (Abbas and Lichtmann, 2005) and play roles in cartilage destruction and bone resorption. The GVT-0 showed the suppresion effect of cytokines level, both IL-1β and TNF-α, in rat ankle tissues induced by CFA. This potency might contribute to the effect of GVT-0 to protect cartilage destruction in CFA-induced arthritis rats.

Curcumin as the analogue of GVT-0 has been extensively studied in term of its mechanism as anti-inflammatory agent. This compound is reported to potently inhibits cytokine-mediated NF-kB activation by blocking a signal leading to IKK activity (Jobin *et al*., 2009).

Since NF-kB plays a central role in mediating proinflammatory gene expression, inhibition on NF-kB activation leads to the inhibition of cytokines synthesis. Shakibaei *et al.*, (2007) also reported that curcumin inhibited the IL-1beta-induced stimulation of up-stream protein kinase B Akt. These events correlated with down-regulation of NF-kappaB targets including COX-2 and MMP-9. Curcumin also reversed the IL-1beta-induced down-regulation of collagen type II and beta1-integrin receptor expression. This indicate that curcumin has anti-inflammatory activity through suppression of NF-kappaB mediated IL-1beta/TNF-alpha catabolic signalling pathways. Based on the mechanism of curcumin, it might be hypothesized that GVT-0 also inhibits activation of NF-kB, which in turn suppresses the expression of various cytokines, including TNF-α and IL-1β. Recently, curcumin is reported to block T cell-activation-induced Ca2 mobilization and thereby prevents NFAT activation and NFAT-regulated cytokine expression (Kliem, *et al*, 2012). Therefore, we might expect that GVT-0 as an analogue of curcumin might have similar mechanism, however, it needs further investigation.

CONCLUSION

The results suggest that GVT-0 is potential to modify the progression of rheumatoid arthritis and can be developed as new drug candidate for rheumatoid arthritis. Further investigation is necessary to elucidate the mechanism of GVT-0 in suppression of the cytokines level and cartilage destruction.

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