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GANODERMA LUCIDUM TOTAL TRITERPENES ALLEVIATE INFLAMMATION AND MODULATE ANTIOXIDANT STATUS IN FREUND'S COMPLETE ADJUVANT-INDUCED ARTHRITIC RATS

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ABSTRACT: *Ganoderma lucidum* total triterpenes were evaluated for anti-inflammatory activity using carrageenan-induced acute and formalin-induced chronic paw oedema models in Swiss albino mice. The total triterpenes, at concentrations 10, 50, and 100 mg/kg b. wt. administered orally, showed 36.96%, 54.35%, 76.09% inhibition of acute and 41.86%, 62.79% and 79.07% inhibition of chronic inflammation. Total triterpenes were also assessed for its effect in attenuating the inflammation and modulating the antioxidant status of Freund's complete adjuvant (FCA) induced arthritis rats. Total triterpenes when administrated orally, at concentrations 10, 50, and 100mg/kg b. wt., showed 58.73%, 73.02%, and 79.37% inhibition of paw oedema in the arthritis rats. Treatment with total triterpenes enhanced the activities of the antioxidant enzymes (SOD, GPx, and catalase) and restored the level of GSH in arthritic animals. Total triterpene treatment also attenuated the enhanced lipid peroxide levels in FCA-induced rats. All these findings support the strong therapeutic potential of *Ganoderma* total triterpenes against inflammation associated-diseases.

INTRODUCTION: Inflammation is a defensive response for eliminating the harmful agent and injured tissues, thereby stimulating tissue repair. When this critical and usually beneficial response occurs in an uncontrolled manner, the result is excessive cellular damage that results in chronic inflammation and the destruction of healthy tissue. Inflammation has been associated with the patho physiology of numerous clinical conditions, including vascular diseases and cancer [1, 2].

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The term arthritis is used to define several forms of painful degenerative and inflammatory joint diseases [3]. Rheumatoid arthritis is a poly-articular joint disease characterized by massive synovial proliferation, sub-intimal infiltration of inflammatory cells, and subsequent destruction of cartilage and bone [4]. It can affect multiple organs, including muscle, bone, and other soft tissues, thus causing varying degrees of joint deformity and instability, leading to painful disabling conditions [3].

Anti-inflammatory and anti-arthritic agents exert various effects that result in the regular activity of the immune system, including the reduction of the number of immune cells. Several natural products are being used as excellent anti-inflammatory agents without worrying about potential side effects. Extracts of *G. lucidum* were found to

possess a significant anti-inflammatory effect against different inflammatory models in animals [5, 6, 7]. Earlier studies conducted in our laboratory also revealed the anti-peroxidative, anti-inflammatory, and anti-mutagenic activities of ethanol extract of the mycelium of *Ganoderma lucidum* [8]. *Ganoderma lucidu*m polysaccharide was found to show significant activity in carrageenan-induced acute and formalin-induced chronic inflammation [9]. *Ganoderma lucidum* polysaccharide was also found to influence the production of pro-inflammatory cytokines in activated rheumatoid synovial fibroblasts [10]. The present work evaluated the anti-inflammatory activity of total triterpenes isolated from *Ganoderma lucidum* using carrageenan-induced acute and formalin-induced chronic paw oedema models. An attempt is also made to assess the effect of total triterpenes in attenuating the inflammation and modulating the antioxidant status of Freund's complete adjuvant (FCA) induced arthritis rats.

MATERIALS AND METHODS:

Isolation of Total Triterpenes: Isolation of the total triterpenes from the fruiting bodies of *G. lucidum* was performed as described earlier [11]. Briefly, a chloroform soluble fraction from the 100% ethanolic extract of *G. lucidum* fruiting bodies was separated and concentrated. The concentrate was then loaded onto a silica gel column and eluted with petroleum ether, chloroform, methanol, and various combinations of these solvents. The fractions that answered the tests for triterpenes 12 were combined and concentrated to give the total triterpenes.

Animals: Male Swiss albino mice used for anti-inflammatory studies and female Wistar rats used for anti-arthritic studies were purchased from Small Animal Breeding Station, Mannuthy, Kerala, India, and were housed in well-ventilated cages under controlled conditions of light and humidity. Animals were provided with standard mouse chow (Sai Durga Food and Feeds, Bangalore, India) and water ad *libitum*. All the animal experiments were carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India, and by the approval of the Institutional Animal Ethical Committee (149/99/CPCSEA dated 23-10-2009).

Anti-Inflammatory Activity of Total Triterpenes:

Carrageenan-Induced Acute Paw Oedema: Male Swiss albino mice weighing 25 ± 2 g (6 weeks old) were divided into five groups of 6 animals. Acute inflammation was produced in all animals by the sub-plantar injection of 20 µl of freshly prepared 1% suspension of carrageenan in normal saline on the right hind paw [13, 14]. Group 1 comprises control animals that received only carrageenan treatment. Animals in Groups 2, 3, and 4 were pre-medicated with total triterpenes (10, 50, and 100 mg/kg body weight) and group 5 with the reference drug diclofenac (10 mg/kg body wt.), orally one hour before carrageenan injection. Group 6 animals were treated with 250µl sunflower oil which was used as the vehicle to administer total triterpenes. The paw thickness was measured using Vernier calipers before and three hours after the carrageenan challenge, from which the degree of oedema formation was calculated.

Formalin-Induced Chronic Paw Oedema: Male Swiss albino mice weighing 25 ± 2 g (6 weeks old) were treated in the same way as in the case of carrageenan-induced paw oedema model [15]. Instead of carrageen, 20 µl of freshly prepared 2% formalin was used as the oedema to the genic agent. Diclofenac (10 mg/kg body weight) was used as the reference drug. The drug treatment was continued for six consecutive days. The paw thickness and the degree of oedema formation were determined regularly for six days after the formalin injection.

Determination of Anti - Arthritic Activity using FCA Induced Arthritis Model: Female Wistar rats weighing 180 ± 20g (15 weeks old) were divided into six groups, consisting of six animals. Arthritis was induced in all groups, except group 1, by the intradermal injection of 0.1ml of Freund's Complete Adjuvant (Genei, Bangalore) into the sub-planar region of the right hind paw [16, 17]. Group 1 include healthy animals that received neither the adjuvant injection nor the drug treatment. Group 2 include control animals that were inoculated with Freund's complete adjuvant but received no drug treatment. Group 3 animals were administered with standard reference drug diclofenac orally at a dosage of 10mg/kg body weight in 0.5 ml distilled water.

Group 4, 5, and 6 animals received 10, 50, and 100 mg/kg body weight total triterpenes orally in 250 µl sunflower oil. Group 7 animals received 250 µl sunflower oil that was used as the vehicle to administer total triterpenes. Diclofenac, total triterpenes, and the vehicle were administered orally one hour before Freund's complete adjuvant (FCA) injection. The oral administration of the total triterpenes and the vehicle were continued, once daily, for 12 days.

The change in paw thickness was assessed by measuring the right hind paw volume using A Vernier calipers just before drug administration and after adjuvant injection. The paw thickness measurement was repeated every three days after adjuvant inoculation until 22nd day. On the 22nd day, *i.e*., at the end of the experimental period, the severity of the secondary lesions was evaluated visually and scored.

The animals were killed under anesthesia and blood samples were collected directly from the heart, and the non-coagulated (heparinised) blood was used for the determination of antioxidant enzyme activities and reduced glutathione level. The serum samples, after precipitating the protein [18], were used for determining the lipid peroxidation levels [19]. The whole blood was used for analyzing Hb content 20, and the erythrocyte lysate was used to estimate the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and reduced glutathione (GSH). The activity of SOD in blood was assessed, after removing the haemoglobin, by the method of Mc Cord and Fridovich [21]. Catalase activity in the blood was measured by the method of Aebi 22, and the glutathione peroxidase activity was determined according to the method of Hafemann *et al.* [23]. Reduced glutathione in blood was determined, after preparing a hemolysate in water, according to the method of Moron *et al.* [24].

Statistical Analysis: All values are expressed as mean ± standard deviation (S.D.). Statistical evaluation of the data was done by one-way analysis of variance (ANOVA) followed by Bonferroni's test using In-Stat Graph Pad software. A 'p' value less than 0.05 was considered significant with respect to the control group.

RESULTS AND DISCUSSION: Acute inflammation induced by carrageenan is the most commonly used method to screen antiinflammatory agents. Effect of total triterpenes on paw oedema volumes of carrageenan and formalin-induced animals at different time intervals are given in **Fig. 1** and **2**, respectively. The total triterpenes significantly inhibited the acute inflammation induced by carrageenan **Fig. 1** and the chronic inflammation induced by formalin **Fig. 2**. in the experimental animals in a dose-dependent manner. The standard drug diclofenac (10 mg/kg b. wt. when administered orally, showed 71.74% inhibition of acute and 76.74% inhibition of chronic inflammation. Whereas the total triterpenes, at concentrations 10, 50, and 100 mg/kg b. wt., showed 36.96%, 54.35%, 76.09% inhibition of acute and 41.86%, 62.79% and 79.07% inhibition of chronic inflammation **Table 1**.

TABLE 1: EFFECT OF TOTAL TRITERPENES ON CARRAGEENAN-INDUCED ACUTE AND FORMALIN-INDUCED CHRONIC PAW OEDEMA IN MICE

Treatments	Increase in paw thickness in cm (% of inhibition)	
	Carrageenan-induced Acute Paw Oedema	Formalin-induced Chronic Paw Oedema
Control	0.115 ± 0.034	0.108 ± 0.028
Diclofenac (10 mg/kg b.wt)	0.033 ± 0.019*** (71.74%)	0.025 ± 0.013*** (76.74%)
TT (10 mg/kg b.wt)	0.073 ± 0.017** (36.96%)	0.063 ± 0.015** (41.86%)
TT (50 mg/kg b.wt)	0.053 ± 0.013*** (54.35%)	0.040 ± 0.026*** (62.79%)
TT (100 mg/kg b.wt)	0.028 ± 0.005*** (76.09%)	0.023 ± 0.010*** (79.07%)
Sunflower oil (250 µl)	0.108 ± 0.013^{ns} (6.52%)	0.098 ± 0.021^{ns} (9.30%)

Values are Mean ± S.D., n=6, ***P<0.001, **P<0.01, nsP>0.05 with respect to control, TT- total triterpenes.

The development of carrageenan or formalin-induced oedema is a biphasic inflammatory process [25-27]. Reactive oxygen species such as superoxide, hydroxyl radical, and hydrogen peroxide play a vital role in the oedema formation by these agents [28 29]. The early phase of this inflammatory process is involved in the production of histamine, leukotrienes, platelet-activating factor, and possibly cyclo-oxygenase products. The delayed phase of the inflammatory response has been linked to

neutrophil infiltration, formation of neutrophil-derived free radicals or oxidants such as H_2O_2, O_2, and OH, and the release of other neutrophil-derived mediators [30, 31]. Inhibitors of reactive oxygen species reduce the severity of inflammation, and hence, the administration of anti-oxidants can have a protective role in these conditions. Most of the anti-inflammatory drugs act as an antioxidants and scavenge free radicals generated during the inflammatory process. The result of the present study indicates that 100 mg/kg b. wt. total triterpenes possess significantly higher anti-inflammatory activity than standard drug diclofenac in attenuating both acute and chronic inflammation. Earlier studies conducted in our laboratory revealed potential *in-vitro* and in vivo antioxidant activities of total triterpenes 11, and this antioxidant power might have helped the total triterpenes in scavenging the free radicals produced during the delayed phase of inflammation.

Freund's complete adjuvants (FCA) are irreplaceable components in the induction of autoimmune diseases and are the most commonly used immune-adjuvants in experimental research [32]. Sub-planar injection of Freund's complete adjuvant in the rat hind paw led to the development of arthritis. Adjuvant arthritis shares many features with human rheumatoid arthritis, and it affects most of the joints and associated tissues [33, 34]. Results of the present investigation reveal that total triterpenes of *G. lucidum* possessed pronounced anti-inflammatory properties in the immunologically mediated inflammatory response induced by injection of Freund's complete adjuvant in rats. The change in the paw oedema volume during 22 days after inoculation of Freund's complete adjuvant is given in **Fig. 3**. Total triterpenes, at concentrations 10, 50, and 100 mg/kg b. wt., when administrated orally, showed 58.73%, 73.02%, and 79.37% inhibition of paw oedema in the arthritis rats **Table 2**. Standard drug diclofenac (10 mg/kg b. wt.), when administered orally, showed 80.95% inhibition of the paw oedema **Table 2**. Adjuvant-induced arthritis in rats is a chronic disease that develops into two phases: acute periarticular inflammation followed by a phase of bone involvement [35]. In the FCA injected animals, the injected right hind paw showed a biphasic inflammatory response with an immediate acute phase at day post-inoculation followed by a delayed chronic phase that reached peak oedema on day 10 of the FCA inoculation. In the control and vehicle group, there was not much reduction in the paw oedema and observed a continued chronic phase.

In the treated groups, the paw thickness was found to be decreased, showing a reduction in the chronic inflammation phase, with the maximum effect on the 22nd day. On the 22nd day, paw volumes of diclofenac and 100 mg/ kg b.wt. total triterpenes treated animals were found to be reverted to the normal levels, indicating their efficiency in curing the inflammation induced by FCA.

Similarly, in the control group, the adjuvant-induced arthritic animals were presented with symptoms such as thickening of hind paws, inflammation on forepaws, redness, and swelling on ears and nose. All these symptoms were markedly reduced in the total triterpenes, and diclofenac treated group **Table 2**.

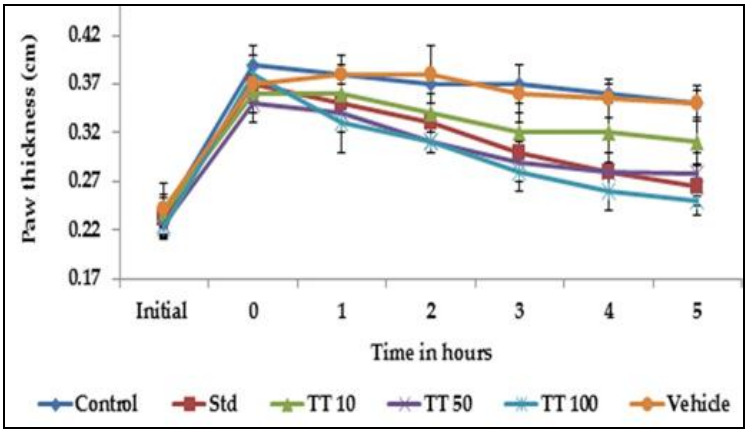

FIG. 1: EFFECT OF TOTAL TRITERPENES ON PAW OEDEMA VOLUMES OF CARRAGEENAN-INDUCED ANIMALS Values are Mean ± S.D., n=6; TT- total triterpenes (mg/kg b.wt.)

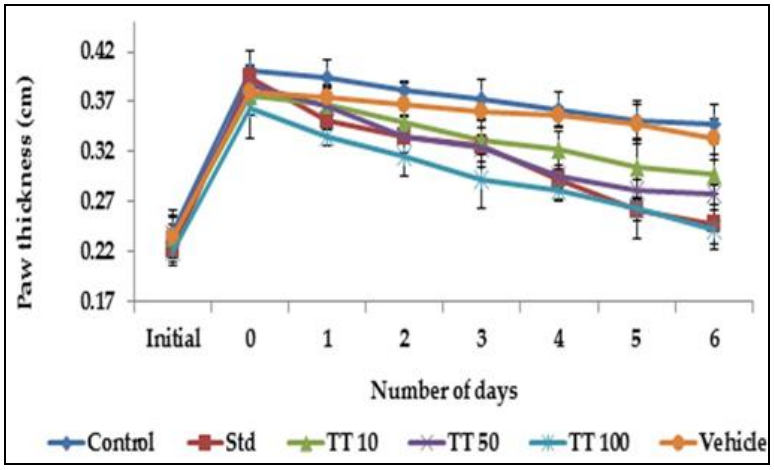

FIG. 2: EFFECT OF TOTAL TRITERPENES ON PAW OEDEMA VOLUMES OF FORMALIN-INDUCED ANIMALS Values are Mean ± S.D., n=6; TT- total triterpenes (mg/kg b.wt.)

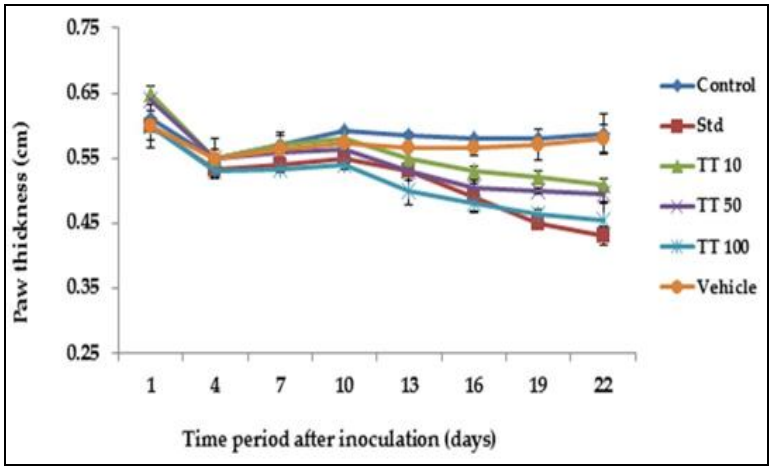

FIG. 3: EFFECT OF TOTAL TRITERPENES ON PAW OEDEMA VOLUMES OF FCA-INDUCED RATS Values are Mean ± SD, n=6; TT- total triterpenes (mg/kg b.wt.).

TABLE 2: EFFECT OF TOTAL TRITERPENES ON PAW THICKNESS AND NUMBER OF SECONDARY LESIONS IN F C A-INDUCED ARTHRITIC RATS

Treatments	Increase in paw thickness in cm (% of inhibition)	Redness on ears	Swelling on nose	Inflammation on forepaws	Inflammation on hind paw other than induced	Inflammation on induced hind paw
Control	0.156 ± 0.029	4/6	2/6	3/6	4/6	6/6
Diclofenac (10 mg/kg b.wt)	0.030 ± 0.014*** (80.95%)	0/6	0/6	0/6	1/6	1/6
TT (10 mg/kg b.wt)	0.065 ± 0.021*** (58.73%)	1/6	1/6	2/6	2/6	3/6
TT (50 mg/kg b.wt)	0.043± 0.030*** (73.02%)	1/6	0/6	2/6	1/6	2/6
TT (100 mg/kg b.wt)	0.031 ± 0.032*** (79.37%)	0/6	0/6	0/6	1/6	1/6
Sunflower oil (250 µl)	0.148 ± 0.034 ^{ns} (6.35%)	4/6	1/6	3/6	4/6	6/6

Values are Mean ± SD, n=6. ***P<0.001, ^{ns}P>0.05 with respect to control, TT- total triterpenes (mg/kg b.wt).

Oxygen-free radicals and H_2O_2 are closely involved in the pathogenesis of rheumatoid arthritis [36]. Superoxide anion or other reactive oxygen species (ROS) liberated by phagocytes are recruited to inflammation sites, which is considered the primary cause of tissue damage associated with many chronic inflammatory diseases [37, 38]. Superoxide radicals and hydrogen peroxide, which in the presence of traces of iron salts found in synovial fluids, interact to form the highly reactive hydroxyl

radical [37, 38, 39]. Granulocytes are sharply increased in number in this disease, and they produce large amounts of O_2^- and H_2O_2 during phagocytosis of immune complexes and other materials. The effect of total triterpenes in neutralizing these free radicals in FCA-induced arthritis rats was evaluated by determining the activities of a non-enzyme anti-oxidant GSH, and the enzymatic anti-oxidants SOD, CAT, and GPx. The activity of enzymatic anti-oxidants and glutathione was decreased in the arthritic control rats compared to the normal rats. But the administration of total triterpenes increased the activity of these enzymes in a dose-dependent manner **Table 3**. The catalase activity in arthritic rats was 71.03 ± 16.06 K/g Hb; while in the 100 mg/kg b.wt of total triterpenes treated group, it was improved to 178.13 ± 21.71 K/g Hb. Similarly, the SOD activity in the control group of animals was 1.95 ± 0.21 U/ml, which was enhanced to 5.74 ± 2.74 U/ml by 100 mg/kg b.wt of total triterpenes treatment. Treatment with the total triterpenes significantly enhanced the glutathione antioxidant system. The activity of GSH in total triterpenes (100 mg/kg b.wt) treated animals was 29.00 ± 14.53nmol/ml of haemolysate. The activity of GPx in blood was also increased to 3.89 ± 0.48 U/ml after treatment with 100 mg/kg b.wt of total triterpenes.

TABLE 3: EFFECT OF TOTAL TRITERPENES ON BIOCHEMICAL PARAMETERS IN BLOOD AND SERUM OF ARTHRITIC RATS

Treatments	SOD (U/ml of haemolysate)	GPx (U/ml of haemolysate)	Catalase (K/g Hb)	GSH (U/ml of haemolysate)	Lipid peroxidation (nmol/ml of protein)
Normal	1.95 ± 0.21 ^{ns}	2.99 ± 0.62^{ns}	71.03 ± 16.06^{ns}	24.88 ± 5.72^{ns}	43.51 ± 4.10^{ns}
Control	1.23 ± 0.46	2.31 ± 0.12	68.08 ± 11.85	18.00 ± 5.79	63.36 ± 10.17
Diclofenac (10 mg/kg b.wt)	1.98 ± 0.26^{ns}	3.01 ± 0.11 ^{ns}	101.44 ± 16.49^{ns}	27.50 ± 3.32^{ns}	51.04 ± 12.18^{ns}
TT (10 mg/kg b.wt)	2.27 ± 1.39^{ns}	3.27 ± 0.58^{ns}	112.12 ± 42.99^{ns}	20.75 ± 4.65^{ns}	47.90 ± 17.5 ^{ns}
TT (50 mg/kg b.wt)	3.22 ± 1.52^{ns}	3.45 ± 1.69 ^{ns}	151.15 ± 69.77**	23.63 ± 17.64^{ns}	37.78 ± 12.37*
TT (100 mg/kg b.wt)	5.74 ± 2.74***	3.89 ± 0.48*	178.13 ± 21.71***	29.00 ± 14.53 ^{ns}	21.28 ± 7.45***
Sunflower oil (250 µl)	1.29 ± 0.60 ^{ns}	2.44 ± 0.32 ^{ns}	76.25 ± 17.54 ^{ns}	16.25 ± 7.97 ^{ns}	67.48 ± 12.93 ^{ns}

Values are Mean ± SD, n=6. ***P<0.001, **P<0.01, *P<0.05, nsP>0.05 with respect to control, TT- total triterpenes (mg/kg b. wt)

ROS is the key to joint destruction [37, 38]. Once generated, these free radicals provoke deleterious effects on various cellular targets, foremost among which are membrane lipids, leading to an extensive process of lipid peroxidation. The increment of lipid peroxide levels in the synovial fluid, serum, and tissue of arthritic animals and patients suffering from arthritis has already been demonstrated [39, 40]. Furthermore, the aggravation of arthritis was associated with the enhancement of lipid peroxidation. The effect of total triterpenes on lipid peroxide formation in the serum of the control and treated groups was evaluated, and the results are given in **Table 3**. The FCA-treated control group showed a substantial increase in the MDA level as compared to the normal groups. But the administration of total triterpenes significantly decreased the serum lipid peroxide level, thereby preventing the joint destruction. Investigations by other researchers also revealed the potential anti-inflammatory action of various extracts of *Ganoderma lucidum* in animals [5, 6, 7, 8, 9]. Both mycelium and fruiting bodies of *Ganoderma lucidum* were found to be highly beneficial against carrageenan-induced acute and formalin-induced chronic inflammation in mice [8, 9]. Moreover, *Ganoderma lucidum* extracts expressed equivalent efficacy as standard anti-inflammatory drugs in reducing inflammation. Additionally, *Ganoderma lucidum* extracts did not show any side effects, such as thymic involution or gastropathy, commonly exhibited by steroids or other non-steroidal anti-inflammatory drugs. All these findings support the strong therapeutic potential of *Ganoderma lucidum* extracts against inflammation associated-diseases.

CONCLUSION: Total triterpenes significantly inhibited inflammation in both carrageens and formalin models, as evident from the decrease in

paw oedema. The anti-inflammatory activity of the total triterpenes at specific doses was significantly higher than the standard drug, diclofenac. It also diminished the inflammation and other arthritic symptoms induced by Freund's complete adjuvant in Wistar rats. The activities of the antioxidant enzymes (SOD, GPx and catalase) and the level of GSH were restored to the normal level in the total triterpenes treated groups. Administration of different doses of triterpenes effectively attenuated the enhanced serum lipid peroxidation due to FCA administration. The results of the present study thus indicate the significant anti-inflammatory and anti-arthritic activity of *Ganoderma lucidum* total triterpenes.

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