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Original Article

In vitro Anti-inflammatory Activity and Acute Oral Toxicity Study of Gold Nanoparticles Generated from Stem Extract of *Tinospora cordifolia*

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ABSTRACT

Introduction: Synthesis of gold nanoparticles (GNPs) from various biological systems have been reported, but amongst them biosynthesis of GNPs from plants is considered as the most suitable method. The aim of this study was to synthesize GNPs using chloroauric acid and hydroalcoholic stem extract (HA) of *Tinospora cordifolia* and to evaluate the *in vitro* anti-inflammatory potential of HA and the synthesised GNP followed by assessing the acute oral toxicity of the prepared herbal GNP. **Materials and Methods:** Anti-inflammatory activity was evaluated by using the *in vitro* human red blood cell membrane stabilization method and gene expression of cytokine TNF-α by isolated human peripheral blood mononuclear cells using RT-PCR. The acute toxicity test was conducted using wistar albino rats. **Results:** The membrane stabilizing effect demonstrated a concentration dependent anti-inflammatory activity of both HA and GNP. GNP synthesized from hydroalcoholic stem extract of *T. cordifolia* inhibited production of proinflammatory cytokine TNF-α by inhibiting genes expression of the cytokine. GNP was found to be non-toxic at a single dose of 2000 mg/kg with no signs of mortality during the 14 days observation period. **Conclusion:** The results of the said evaluation proved that formation of GNP enhanced the anti-inflammatory activity of *T. cordifolia*. The acute oral administration of GNP was found to be safe and not toxic. Hence, the GNP can be utilized for treatment of diseases associated with imbalanced cytokine production as in anti-inflammatory pharmaceutical formulations.

Keywords: *Tinospora cordifolia*, Gold nanoparticles, *In vitro* anti-inflammatory activity, Tumor necrosis factor – α, Acute toxicity.

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INTRODUCTION

Inflammatory disorders can emerge if the control of cytokines is disrupted in any way. As a result, discovering inhibitors that can block inflammatory cytokines becomes a critical step in reducing inflammation. Amongst these cytokines, the pro-inflammation factor Tumor necrosis factor -α (TNF-α) plays a crucial role in many immune and inflammatory processes, such as proliferation, apoptosis, necrosis, and cell survival.[1-2] TNF signalling abnormalities cause rheumatoid arthritis, psoriasis, Crohn's disease, and atherosclerosis. Because of TNF-α`s relevance, controlling its activity has become a key method in the treatment of inflammation related disorders. The identification of compounds that may act as antagonists of this cytokine by oral route is currently in demand. Plant source might provide to be an alternative and cost-effective drug, that can regulate TNF- α levels.

Green synthesis of GNPs using plant extract is recommended as an eco-friendly alternative to biological and chemical methods that reduces the maintenance of septic environment and eliminates the generation of toxic byproducts.[3] Herbal gold nanoparticles synthesized from different plant extracts have been tested for various activities like *in vitro* antioxidant,[5] *in vitro* cytotoxicity,[4] anti-cancer[5] and antimicrobial activities.[6]

Tinospora cordifolia, which belongs to Menispermaceae family is a large, deciduous, climbing shrub found throughout India and also in Sri Lanka, Bangladesh, and China. It is one of the most important herbs of Ayurveda, designated as Rasayana. It has been used to treat fever, jaundice, dengue, gout, skin diseases, diabetes and rheumatoid arthritis.[7] Analgesic, anti-inflammatory and anti-pyretic[8-10] activities of various extracts of *T. cordifolia* have been studied in different animal models but there is no work done using GNP synthesized from this herb.

There is paucity of data on animal toxicity studies of synthesized herbal GNPs. For *In vitro* studies, one requires small quantity of synthesized GNPs but in order to conduct animal studies, more quantities would be needed, which makes the process time-consuming, laborious and expensive, which could be the reason why not much research has been done on animal models using herbal GNPs. The present investigation is planned for the first time with the objective to evaluate the acute oral toxicity effects of GNP prepared from *T. cordifolia* in albino wistar rats as per OECD guideline 423 as well as to evaluate the *in vitro* anti-inflammatory potential of the said extract and the synthesised GNP from the extract.

MATERIALS AND METHODS

Preparation of *Tinospora cordifolia* stem extract

Fresh stem of *Tinospora cordifolia* were collected from Mumbai, India and authenticated by Dr. Rajendra D. Shinde, Director of Blatter Herbarium St. Xavier's College, Mumbai (Specimen number P/D/2868/2018). Fresh stems were cut into small pieces, washed with water and dried in tray drier at 50vC for a month. The dried stems were milled to a coarse powder, 1Kg of the dried powder was extracted with hydroalcoholic solvent by maceration at room temperature. The rotavap was used to remove the alcohol from the filtered hydroalcoholic stem extract (HA)

and the aqueous extract thus obtained was dried to a semisolid mass which was stored in a refrigerator at 4°C for further studies. For the rest of the work distilled water (DW) was used.

Synthesis of gold nanoparticles

1mM gold chloride solution and 1.25% of HA was prepared in DW. Filtered extract and gold chloride solution was mixed in 1:9 ratio and kept on a magnetic stirrer at 400 rpm for 3 hr at room temperature for synthesis of GNP. The change in the color of the solution from brown color to violet color indicated the reduction of Au^{3+} ions to Au0. The solution was centrifuged at 10,000 rpm for 15 min. Supernatant was discarded and pellets were washed thrice with DW.[11] Finally, the violet-colored GNP pellets were dried at 37°C and stored for further studies.

Assessment of *in vitro* Anti-inflammatory Activity Membrane Stabilization Method

Preparation of human red blood cells (HRBC) suspension

The Blood was collected from healthy human volunteer who has not taken any Non -Steroidal Anti-Inflammatory Drugs (NSAIDs) for 2 weeks prior to the experiment and transferred to the tubes containing an anti-coagulant. The tubes were centrifuged for 10 min at 3000 rpm and washed three times with sterile saline solution. The volume of blood was measured and reconstituted as 10% v/v suspension with sterile saline solution.

Hypotonicity-induced hemolysis

Different concentration of HA extract and GNP at a volume of 0.5 ml (0.0625-1 mg/ml) and control (Saline) were separately mixed with 0.475 ml of hyposaline (0.36%) and 0.025 ml of HRBC suspension. Standard drug used was diclofenac sodium. All the assay mixtures were incubated at 37°C for 30 min and centrifuged at 3000 rpm.[12] Hemoglobin content of the decanted supernatant liquid was estimated by a spectrophotometer at 560 nm. The percent hemolysis was estimated by assuming the hemolysis produced in the control as 100%.

All the experiments were done in triplicates.

$$\text{Percentage protection} = 100 - \left(\frac{\text{OD sample}}{\text{OD control}}\right) \times 100$$

TNF- α Inhibition Assay

PBMC preparation and cell culture

In order to isolate human peripheral blood mononuclear cells (PBMC), blood was collected from healthy human volunteers (age ranging 22–45). Informed consent was obtained before a 15 ml volume of peripheral blood was collected by venipuncture in sterilized ethylenediamine tetra-acetic acid (EDTA) tubes. PBMCs were separated by Ficoll-Paque density gradients (Pharmacia Biotech, Uppsala, Sweden) and cells from the interface were washed twice with calcium and magnesium free phosphate buffer saline (PBS) and seeded at a density of 1×10^4 cells/ml per well in 24-well microtiter plates in triplicates. Separated PBMCs were incubated in RPMI-1640 media supplemented with sodium bicarbonate, L-glutamine-penicillin-streptomycin solution (200 mM L-glutamine, 10,000 U penicillin and 10 mg streptomycin/mL in 0.9% NaCl) and 10% v/v FCS.[12-13] Cells were maintained at 37°C for 4, 6 and 20 hr at 5% CO_2 and the levels of secreted TNF-α mRNA expression were determined as described below.

Gene expression quantification by RT-PCR analysis

For the determination of cytokine mRNA expression, cells were seeded at a density of 1×10^4 cell/ml in 24-well plates. PMA (100 ng/ml) and Vitamin D3 (0.1 M) were used to activate the cells. *Escherichia coli* lipopolysaccharide (LPS) was used as a positive control stimulus for cytokines induction at a concentration of 5 µg/ml (*E. coli* LPS 026: B5, Sigma). Twenty-four hours after cell activation, LPS (5 µg/ml) stimulated PBMCs were treated with HA-extract (50 µg/ml) and GNP (50 µg/ml). The expression of TNF-α was tested after 48 hr.

Total RNA was obtained from cells using the Rneasy Plus Mini Kit (QIAGEN) according to manufacturer's instructions, and immediately frozen at -80°C until use. For RT-qPCR, DNase-treated RNAs were used to synthesise cDNA with the Transcriptor First Strand cDNA Synthesis Kit using random hexamers as specified by the manufacturer (Thermo Fischer Scientific Maxima First Strand cDNA Synthesis Kit). After 48 hr of treatment RNA was isolated from PBMCs (1×10^4 cell/ml). All RNA samples from a single experimental setup were reverse transcribed simultaneously and in triplicates, to minimize variation in the reverse transcription reaction. qPCR was performed on ABI Step One Plus system using SYBR Green chemistry PCR (Applied Biosystem).[13-14] PCR amplifications and Advanced relative quantification analysis were achieved using a Light Cycler 480 instrument (Roche Applied Science) with software version LCS480 1.5.0.39. All reactions were performed in triplicates in a final 20 µl volume with 2.5 mM $MgCl_2$, 0.2 µM of each primer and 2 µl cDNA. For a single reaction, total RNA (0.1 g) was used. The amplification of cytokine cDNA was accomplished utilizing PCR primers obtained from Aragene Company (Aragene, Riyadh, Saudi Arabia). The primers used for TNF- α a sense and antisense, respectively, were CCA-GGC-AGT-CAG-ATC-ATC-TTC and GAT-GGC-AGA-GAG-GAG-GTT-GAC. Conditions for RT-PCR were 45 cycles (95°C for 15 s, 60°C for 20 s and extension time at 72°C for 30 s). As an internal control for the RT-PCR assay, the housekeeping gene b-actin was utilized. The primers used for b-actin were AGA-GCT-ACG-AGC-TGC-CTG-AC and AGC-ACT-GTG-TTG-GCG-TAC-AG. Conditions for RT-PCR were 45 cycles (95°C for 15 s, 60°C for 20 s and extension time at 72°C for 30 s).[15] All experiments were done in triplicates. The results were analysed by relative quantification, using Δ Ct method.

Acute oral toxicity study

Experimental animals

All animal experiments were performed according to ethical guidelines suggested by the institutional animal ethics committee (IAEC). The study was carried out at Bombay Veterinary College, Parel. The protocol was approved in IAEC protocol no: BVC/IAEC/08/2019. From the *in vitro* cytotoxicity test done using MTT dye conversion method by our group previously, it was observed that the synthesized GNP were non-toxic and biocompatible. Based on the acute toxicity study reported on *T. cordifolia* using the decoction, whole plant powder,[16] and the GNP being green synthesized the animal ethics committee approved the protocol to dose 2000mg/kg body weight of the synthesized GNP to all the 6 animals of the treatment group. Adult female Wistar albino rats weighing about 150 to 250 g were used for the experiment. The rats were kept individually in properly numbered large polypropylene cages with stainless steel top grill having facilities for pelleted feed, corncob was used as bedding material and changed twice a week. The animals were kept in a well-ventilated animal house under natural conditions in a 12 hr light-dark cycle at 28°C ± 2°C, and they were acclimatized to laboratory settings for 7 to 10 days prior to the start of the experiment. The animals were fed with standard pelleted diet and water as and when required

Experimental design

The animals were acclimatized to the laboratory conditions prior to the test. Before the test, animals were randomized and assigned to the treatment groups. The animals were randomly divided into two groups each containing six rats as shown in Table 1. They were identified by the markings using a yellow stain. Group I was vehicle control and Group II

Table 1: Group Distribution for acute Toxicity in Albino Wistar Rats.

Group	Treatment	Route of administration	No. of Animals
Group I	Vehicle Control	Oral	6
Group II	GNP 2000mg/kg	Oral	6

was administrated 1ml of aqueous gold nanoparticle solution in a single dose of 2000 mg/kg via oral gavage.[17] Individual animals were observed at 30 min, 1,2,3 and 4 hr post dosing on day 0(day of dosing) for clinical signs and symptoms. Subsequently, all the animals were observed once a day for any behavioural changes and mortality during the 14 days observation period. Body weight was recorded on day 0 and on day 14.

Cage side observations recorded were changes in fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system regulated symptoms, and somatomotor activity with many other day to day life behavior patterns etc.

Mortality, general signs, and physical observation of the animals for symptoms such as diarrhea, lethargy, depression, breathing difficulty, shuffling, skin and fur problems, salivation, tremors, tears, equilibrium sensation, excitement, convulsions, dermatitis, bristling of fur, excess salivation, in any group. The appearance of urine and feces of treated rats, including color and odor, were observed.

RESULTS

In vitro Anti-inflammatory Activity

Membrane stabilization method

The results of anti-inflammatory activity determined by the human red blood cell membrane stabilization test are shown in Figure 1. Both HA and GNP demonstrated a concentration dependent anti-inflammatory activity and the protection percent increased with increase in concentration of the samples. The membrane stabilizing effect of HA ranged from 8.4 ± 1.28% to 60± 0.8% inhibition of RBC hemolysis and for GNP it was in the range of 27± 1.65% to 73± 0.79% inhibition of RBC hemolysis for concentrations ranging from 0.0625 mg/ml to 1mg/ml compared to that of standard. Diclofenac sodium exhibited 95% protection at 100 µg/ml concentration. The IC_{50} value of HA and GNP were 0.727 mg/ml and 0.421 mg/ml respectively, whereas the standard diclofenac sodium has an IC_{50} of 0.091 mg/ml as shown in Figure 2. It is clear from the data that GNP showed greater anti-inflammatory property compared to HA.

TNF- α inhibition assay

Data presented in Figure 3 show the effect of same concentration of HA extract and GNP on TNF-α gene expression in PBMC`s from healthy donor at 48 hr of incubation as quantified by real time Q-PCR. Treatment of GNP and HA extract resulted in a significant downregulation of cytokine genes expression. At 48 hr HA extract at 50 ppm inhibited the TNF-α expression 1.84 fold ($p<0.05$) whereas GNP at 50 ppm inhibited the TNF-α expression 7.73 fold ($p<0.001$) compared to LPS stimulated control. This data suggests that a significant inhibitory effect of GNP on TNF-α expression compared to HA extract.

Acute oral toxicity study

The study results of "acute oral toxicity" indicates that under experimental set up and laboratory environment, the GNP was found to be non-toxic at the dose level of 2000 mg/ kg by oral route. The rats were generally active and showed no visible signs of illness or toxicity. Body weight gain was also observed as shown in Figure 4, when compared with before and after treatment of the observed groups. The graph reveals that there was a significant increase in body weight before and after treatment but both the groups showed similar increase suggesting no abnormal increase in the body weight. No mortalities were recorded throughout the study period. The treated animals survived throughout the study period and did not show any clinical signs of toxicity at the tested dose level. The summary of clinical signs of animals is presented in Table 2.

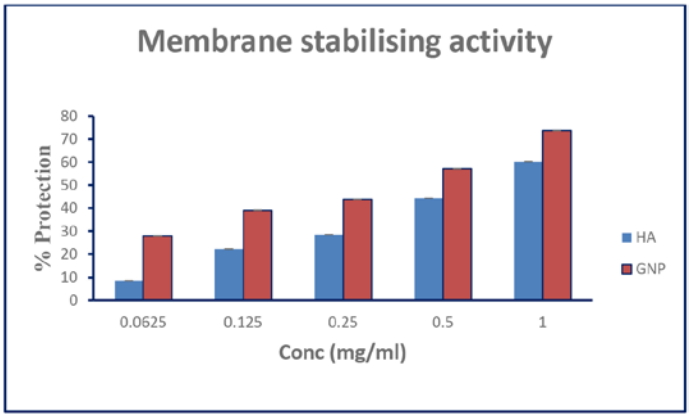

Figure 1: Effect of HA and GNP on hypotonic solution induced hemolysis of erythrocyte membrane. Each value is expressed as mean ±SD (n=3).

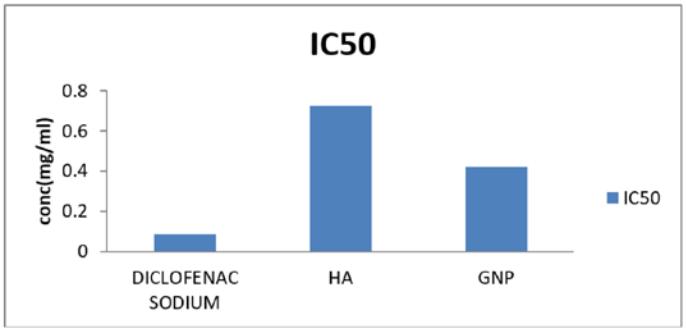

Figure 2: IC_{50} values of standard diclofenac sodium, HA and GNP.

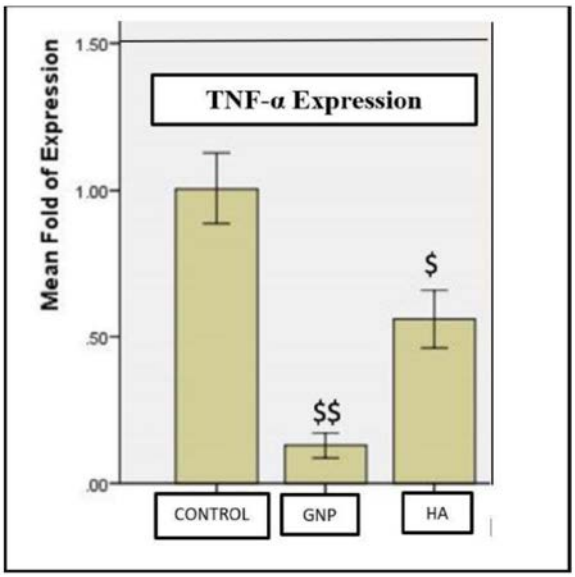

Figure 3: Mean fold of expression of TNF-α when PBMC`s were treated with GNP and HA by RT-PCR; The results are expressed as means ± SD. $^{\$}$ Indicates significant difference from the control (n=3, $^{\$}$ $p<0.05$ and $^{\$\$}$ $p<0.001$).

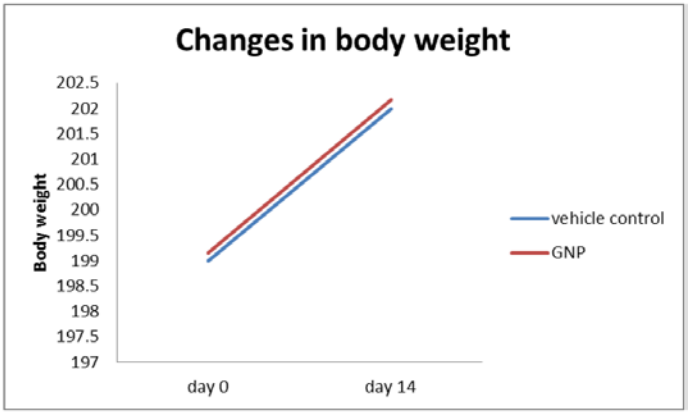

Figure 4: Changes in the Body Weight of the RATS Treated with GNP and Vehicle Control.

Table 2: Effect of Aqueous solution of GNP on Acute oral Toxicity test in Albino Wistar Rats.

Observations	24 hr	7th day	14th day
Mortality	Nil	Nil	Nil
Eyes	NAD	NAD	NAD
Respiratory rate	NAD	NAD	NAD
Salivation	NAD	NAD	NAD
Mucous membrane	NAD	NAD	NAD
Motor activity	NAD	NAD	NAD
Behavior pattern	NAD	NAD	NAD
Skin and fur	NAD	NAD	NAD
Lethargy	NAD	NAD	NAD
Diarrhea	NAD	NAD	NAD
Food intake	Normal	Normal	Normal
Water intake	Normal	Normal	Normal

NAD - No Adverse effects Detected

DISCUSSION

The GNP synthesized using stem extract of *T. cordifolia* in our lab gave the following characterization results: UV-VIS spectrum of the aqueous medium containing, gold nanoparticles showed a peak at 546 nm. The hexagonal shaped nanoparticles were well dispersed with particle size ranging from 30-60 nm, that were confirmed by TEM and SEM respectively also reported by Abbasi *et al.*[11] FTIR showed shift in position and intensity of the peaks. HPTLC screening showed that even after forming gold nanoparticles, it retains most of its phytochemical constituents. *In vitro* stability studies have confirmed that gold nanoparticles are stable in biological fluids at physiological pH and also in salt solutions. XRD studies confirmed crystalline nature of the synthesized nanoparticles. Zeta potential value of the synthesized gold nanoparticles is -29 mV at 25° showing good stability of nanoparticles.

In the membrane stabilization method to assess the *in vitro* anti-inflammatory activity, HA as well as GNP failed to show promising hemolytic effect on HRBC membrane at all doses used (0.0625-1 mg/ml), both HA and GNP showed significant anti-inflammatory activity in a concentration dependent manner. The results of the present investigation showed the anti-inflammatory property of HA and GNP, which is comparable to some of the studies which was conducted on the extract of other plants such as *B.monnieri*,[18] *T. chebula*[19] etc. using HRBC membrane stabilization method. In the study conducted in our lab, *In vitro* antioxidant study showed that 2, 2-diphenyl-1-picrylhydrazyl activity increased in a dose dependent manner for both HA and GNP. GNP exhibited a higher membrane protection activity of 73% as compared to that of HA which showed a 60% protection at a concentration of 1 mg/ml as shown in Figure 1. Thus formation of GNP enhanced the anti-inflammatory properties of *T. cordifolia*. The standard drug diclofenac sodium was found to possess the best IC_{50} value followed by GNP and HA as shown in Figure 2. The enhanced anti-inflammatory effect of GNP might be the result of active physicochemical interaction of gold atoms with the functional groups of *T. cordifolia*. Thus the *in vitro* results suggests that GNP could become a potential therapeutic agent to treat human inflammatory diseases.

The TNF- α inhibition assay was performed to assess the pharmacological effects of HA and GNP on the LPS stimulated production of pro-inflammatory cytokines in human PBMCs *in vitro*. In recent years, various herbal-derived extracts and phytochemicals have been found to modulate the production of pro-inflammatory mediators. Thus, herbal-derived inhibitors of these mediators are highly appreciated as potential candidates for development of new anti-inflammatory drugs with reduced side effects. *T. cordifolia* is widely used in folk medicine and Ayurveda as an anti-inflammatory agent. Previous studies have indicated for the presence of various phytochemicals in *T. cordifolia* extract that have anti-inflammatory activity.[20-22] The bioactive fraction of *T. cordifolia* in methanol was found to inhibit TNF-α and IL-1β production in LPS-treated dendritic cell suspensions as compared to standards.[23] Remarkable immunomodulatory activity of methanolic extracts of *T. cordifolia* grown on *Azadirachta indica* and *Mangifera indica* on PBMC at 50–100 µg/ml concentration have been reported.[24]

The present study found that GNP synthesized from HA of *T. cordifolia* inhibited production of proinflammatory cytokine TNF -α by inhibiting genes expression of the cytokine and proved that formation of GNP enhanced the anti-inflammatory activity of *T. cordifolia* as shown in Figure 3. This study clearly revealed that HA and GNP inhibited the LPS stimulated upregulation of proinflammatory cytokines at the same dose of 50 ppm, without causing any significant cytotoxic effects. This anti-inflammatory activity was postulated due to the significant inhibition of TNF -α. This is the first report on the anti TNF-α activity of GNP of *T. cordifolia* although various extracts of the herb has been reported to possess anti-inflammatory activity. Thus, the inhibitory effects of GNP on the expression of inflammatory mediators comprise one of the mechanisms behind its anti-inflammatory effects, suggesting that GNP might be applied with in novel approaches for boosting the rheumatoid arthritis anti-inflammatory activity. Hence further analysis of the effect of GNP on other mediators of inflammation will help in the molecular profiling of the anti-inflammatory properties of GNP synthesized from *T. cordifolia*

In acute oral toxicity study no mortality and morbidity or any signs of behavioural changes or toxicity were observed throughout the 14-day period after single oral administration of violet coloured GNP at the dose of 2,000 mg/kg body weight. Morphological characteristics fur, skin, eyes, and nose appeared normal. No tremors, convulsion, salivation, diarrhoea, lethargy or unusual behaviours such as self-mutilation, walking backward and so forth were observed on oral administration of GNP as shown in Table 2. Gait and posture, reactivity to handling or sensory stimuli, grip strength was normal.

Oral administration of GNP showed significant increase in body weight 14 days post administration, but the weight gain was similar to the control group as shown in Figure 4. From the results of the current study, the overall percent body weight gain in treated rat was found to be 1.5% at the end of 14 days, suggesting normal increase in body weight. The body

weights of the control and treatment groups did not differ significantly. When compared to the control groups, the appearance of treated rats' urine and faeces, including colour and odour, was identical.

The multi-target responses of herbal drugs are proven to be beneficial in chronic conditions such as diabetes, rheumatoid arthritis, cancer and so forth. Toxicity testing is required, especially for medications that will be used in chronic diseases. The findings of the current studies did not reveal any major abnormal behavioural or clinical signs relevant to the screened products at the employed dose level of 2000 mg/kg. Therefore, the LD_{50} value for GNP was estimated to be more than 2000 mg/kg. This indicated that the oral administration of aqueous GNP could be considered safe and nontoxic.

CONCLUSION

For the control and treatment of many ailments, there has been a growing interest in the study of therapeutic potentials of natural products derived from plants. In the present work, anti-inflammatory activity was evaluated by *in vitro* human red blood cell membrane stabilization method, which showed significantly higher anti-inflammatory activity at increasing concentration of GNP compared to that of the extract. Further expression of cytokine TNF-α by total peripheral blood mononuclear cells suggested that the anti-inflammatory effects of GNP is mediated via suppressing the production of TNF-α gene expression levels in PBMCs which should be confirmed by using *in vivo* anti-inflammatory study models. The results of *in vivo* acute toxicity study clearly showed the non-toxic nature of gold nanoparticles synthesized from *T. cordifolia* at the tested dose level of 2000 mg/kg. No death or signs of toxicity were observed in rats treated with aqueous solution of GNP following dosing and during the observation period of 14 days thus establishing its safety in use. The LD_{50} value can be considered to be more than 2000 mg/kg. It was concluded that further anti-inflammatory studies in animal models and chronic toxicity studies in *in vivo* animal models are warranted to complete the safety profile of this herbal gold nanoparticle.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

GNP: Gold nanoparticles; **HA:** Hydroalcoholic extract; **PBMC:** human peripheral blood mononuclear cells; **TNF-α:** Tumor necrosis factor -α.

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