

ANTI-INFLAMMATORY AND ORAL TOXICITY ASSESSMENT OF R-38™: A TRADITIONAL HERBAL SUPPLEMENT FOR ARTHRITIS

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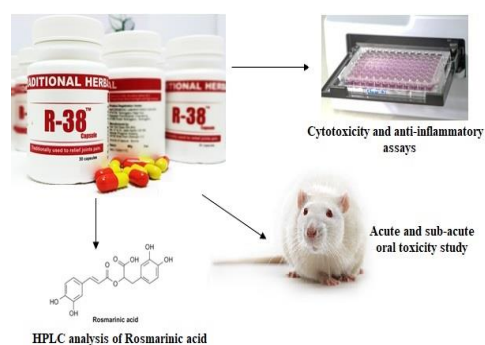
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Graphical abstract



Abstract

R-38™ is a traditional herbal supplement for treating arthritis-related conditions. High-performance liquid chromatography (HPLC) analysis was performed for identification and quantification of rosmarinic acid in the R-38™ water extract. The anti-arthritic potential of the R-38™ water extract was investigated by measuring the production of IL-6 and TNF- α in inflamed cells. Xanthine oxidase (XO) and cyclooxygenase-2 (COX-2) inhibition assays were also conducted. The cytotoxic effect of R-38™ water extract was investigated on HSF1184 cell line. Acute and subacute oral toxicity studies were conducted on female Sprague-Dawley (SD) rats. The rosmarinic acid was identified at 1.208 min (3.61 %w/w). The inflamed cells showed a decrease in the production of IL-6 (55.9%) and TNF- α (52.13%). The COX-2 and XO enzymes were moderately inhibited by R-38™ water extract. The cytotoxicity analysis showed no cytotoxic effect on the cell. The acute and subacute oral toxicity studies revealed no mortality and normal body weight at all doses. There were no significant differences ($p > 0.05$) in organ weight, hematological and biochemical parameters, and histology of liver and kidneys with the control group. In conclusion, R-38™ water extract exhibited no toxic effect orally and may possess potential therapeutic properties against pro-inflammatory diseases including arthritis.

Keywords: Botanical medicine, R-38™, anti-inflammatory, oral toxicity, rosmarinic acid, arthritis

Abstrak

R-38™ adalah suplemen herba tradisional untuk merawat keadaan yang berkaitan dengan arthritis. Analisis Kromatografi Cecair Berprestasi Tinggi (HPLC) telah dilakukan untuk pengenalpastian dan kuantifikasi asid rosmarinik di dalam ekstrak air R-38™. Potensi *anti-arthritic* ekstrak air disiasat dengan mengukur penghasilan IL-6 dan TNF- α dalam sel-sel yang meradang. Asai perencatan *xanthine oxidase* (XO) dan *cyclooxygenase-2* (COX-2) juga dilakukan. Kesan sitotoksik ekstrak air R-38™ disiasat pada garis sel HSF1184. Kajian ketoksikan oral akut dan subakut dilakukan pada tikus Sprague-Dawley (SD) betina. Asid rosmarinik dikenal pasti pada 1.208 minit (3.61% w/w). Sel-sel yang meradang menunjukkan penurunan dalam penghasilan IL-6 (55.9%) dan TNF- α (52.13%). Enzim COX-2 dan XO direncat secara sederhana oleh ekstrak air R-38™. Analisis sitotoksik menunjukkan tiada kesan sitotoksik kepada sel. Kajian ketoksikan oral akut dan subakut menunjukkan tiada kematian dan berat badan normal pada semua dos. Tiada perbezaan yang signifikan ($p > 0,05$) pada berat organ, parameter hematologi dan biokimia, dan histologi hati dan ginjal dengan kumpulan kawalan. Kesimpulannya, ekstrak air R-38™ tidak menunjukkan kesan toksik secara oral dan mungkin mempunyai sifat terapeutik yang berpotensi terhadap penyakit pro-radang termasuk arthritis.

Kata kunci: Ubat botani, R-38™, anti-radang, ketoksikan oral, asid rosmarinik, arthritis

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1.0 INTRODUCTION

Rheumatoid arthritis (RA) is the most prevalent form of inflammatory arthritis. It is a disease of unknown etiology that has affected approximately 1% of the population worldwide and is correlated with work disability among adults of all ages. The systemic nature of RA involves inflammation of many joints, mainly the lining of synovial joints [1]. In RA symptomatic treatment, non-steroidal anti-inflammatory drugs (NSAIDs) are the most prescribed drugs. However, there are limitations and risks associated with the use of these drugs. Hence, the use of alternative therapies involving medicinal herbs is markedly increasing. A previous report stated that more than half of arthritis patients are using complementary and alternative medicine (CAM) [2]. Most of the developing countries (80% of the population) are still sought for traditional herbal medicines in treating their illnesses as estimated by World Health Organization (WHO) [3].

Traditional herbal medicine has been practiced since ancient times and the growing interest among people towards herbal medicine stipulates the need for thorough investigations into the safety and efficacy of traditional medicines. Though most of these formulations are commonly used by traditional medicine practitioners, yet they still lack scientific evidence. One such Malay traditional herbal medicine, R-38™ formulation, is used for its anti-inflammatory property, managing arthritic conditions, and improving health with a recommended dose of 300 mg per capsule, once to twice a day. The R-38™ comprises of galangal (*Alpinia galanga*), temulawak (*Curcuma xanthorrhiza*), cat's whiskers (*Orthosiphon stamineus*), white turmeric (*Curcuma zedoaria*), and

prickly-leaved elephant's foot (*Elephantopus scaber* L.). The R-38™ has been approved by the National Pharmaceutical Regulatory Agency (NPRA), Ministry of Health Malaysia, as traditional medicine (MAL 1703501 ITC) used to relieve joints pain.

The herbal ingredients of R-38™ are locally available in Malaysia and well known among traditional medicine practitioners as remedies for arthritis-related conditions [4, 5, 6]. They have been reported to possess bioactivities such as anti-inflammatory [4, 7-14], hepatoprotective [15], and anti-arthritic [7, 11, 14, 16-19]. They were also reported to have no toxicity effects and considered as safe [9, 10, 19-22].

One of the ingredients of R-38™ is *O. stamineus*. It has been reported to contain rosmarinic acid, which is a derivative of caffeic acid [23]. Scientific studies have reported on the anti-inflammatory potential of rosmarinic acid in managing inflammatory diseases. The in vivo treatment with 50% ethanol leaves extract of *O. stamineus* was reported to exhibit dose-dependent inhibitory effects on the rat hind paw edema by significantly decreased synovial hyperplasia, inflammation, and cartilage erosion. It also showed potent inhibition to pro-inflammatory mediators such as Interleukin-1 β (IL-1 β), tumor necrosis alpha (TNF- α), cyclooxygenase-1 (COX-1), and COX-2 in vitro [16]. Recently, isolated rosmarinic acid from pomegranate peel was reported to significantly reduce arthritis index score, paw volume, and production levels of TNF- α in Freund's complete adjuvant (FCA)-induced arthritis rats [18].

R-38™ presently has no sufficient evidence-based data to support its anti-inflammatory activities, safety, and effectiveness of the formulation. The present study aimed to evaluate the therapeutic potential of

R-38™ water extract by evaluating its anti-inflammatory activities and toxicity effects by in vitro and in vivo studies. The rosmarinic acid in the R-38™ water extract was also identified and quantified by HPLC analysis.

2.0 METHODOLOGY

2.1 Chemicals and Reagents

Chemicals used for HPLC analysis such as acetonitrile (HPLC grade), methanol (HPLC grade), and ethanol 95% (AR grade) were purchased from Sigma-Aldrich (St. Louis, MO). The standard active compound, rosmarinic acid (SC-202796A) was purchased from Santa Cruz Biotechnology (Dallas, TX). For cytotoxicity study, bacterial lipopolysaccharide (LPS, serotype: 0111:B4, L5293), dispase, collagenase (Type 1), glutamine, penicillin, dissection balanced salt solution (DBSS), fetal bovine serum (FBS), and Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA). Human Tumour Necrosis Alpha (TNF- α) enzyme-linked immunosorbent assay (ELISA) kit (Catalogue No: RAB0307) and Human Interleukin-6 (IL-6) ELISA kit (Catalogue No: RAB0476) were purchased from Sigma-Aldrich (St. Louis, MO). For the animal study, 10% neutral buffered formalin solution, 0.9 % normal saline, toluene (AR grade), xylene (AR grade), absolute ethanol, paraffin wax, Haematoxylin and Eosin (H&E) solution were procured from Tricell Bioscience Resources, (Selangor, Malaysia). Anaesthetic, KTX (Ketamine-Xylazine/Zoletil) mixture was obtained from the Laboratory Animal Resource Unit (LARU) at Universiti Kebangsaan Malaysia (Selangor, Malaysia).

2.2 Plant Materials and Extraction

The coarsely ground of R-38™ raw material was purchased from Naturemedics Laboratories Sdn. Bhd. (Kuala Terengganu, Malaysia). The formulation was kept in an air-tight container and stored at 4 °C until further used. The formulation was soaked in filtered water (1:10) at 55 °C and stirred gently for 3 h. The extract was filtered and left to dry completely in the freeze-dryer (Labconco Freezone 4.5 Freeze Dryer, Labconco Corporation, Kansas City, MO) for 7 days. The freeze-dried extract was stored at 4 °C in an air-tight container until further used.

2.3 Cell Culture

Human skin fibroblast (HSF1184) cell line (Sigma-Aldrich) sample was rinsed in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) with 2.4 U/mL dispase, followed by overnight incubation (4 °C) for facilitating the separation of the epidermis from the dermis. The separated dermal part was

washed in dissection balanced salt solution (DBSS), minced, and placed into 0.3% collagenase (3–4 h) until fully digested at 37 °C. The HSF1184 was washed with DBSS and cultured in DMEM/F12 supplemented with 10% FBS. The cells were seeded at 1×10^4 cells/cm² in a humidified atmosphere of 5% CO₂ at 37 °C.

2.4 Cytotoxicity Assay on HSF1184 Cell Line

MTT (3-(4, 5-dimethylthiazolyl)-2-yl-2,5-diphenyltetrazolium bromide) assay was carried out to determine the 50% inhibitory concentration (IC₅₀) of water extract of R-38™ on HSF1184 cell. The cell viability was tested with the trypan blue exclusion method using Neubauer Improved bright-line hemocytometer (FORTUNA®, Germany). The assay was done following the method by Patel *et al.* (2009) [24] with modification. Preparation of the sample stock solution was done by dissolving 55 mg of the sample in 550 μ L of the medium. The MTT assay was carried out with seven different concentrations of R-38™ water extract from 10^{-1} - 10^5 μ g/mL along with control in triplicates. The cells were seeded at 1×10^4 cells/mL in a 96-well plate. MTT solution (100 μ L) was added into wells and incubated for 4 h at 37 °C. The MTT solution was then discarded and 100 μ L of MTT solvent (0.1 N HCL-Isopropanol solution) was added into all wells. The number of formazan crystals was recorded using microplate reader Bio-Tek, Model ELX 808 (Bio-Tek, Winooski, VT) at 570 nm and a reference wavelength of 630 nm. Based on the test finding, concentrations were selected to be used in the anti-inflammatory studies of the R-38™ water extract on HSF1184 cell-induced with lipopolysaccharide (LPS).

2.5 Quantification of Anti-inflammatory Activities

The anti-inflammatory activity of the R-38™ water extract was investigated on lipopolysaccharide (LPS) induced-HSF1184 cells through the production of IL-6 and TNF- α . Cellular viability was measured using MTT assay while TNF- α and IL-6 production were measured using Human TNF- α ELISA kit and Human IL-6 ELISA kit, respectively. The HSF1184 cells were seeded (1×10^4 cells/mL) on three plates of 96-well plates for MTT assay, IL-6, and TNF- α ELISA tests. Four series of each plate were grouped as a positive control, negative control (cells induced with 1.5 μ L/mL LPS), treatment I (LPS-induced cells and treated with 10^4 μ g/mL water extract), and treatment II (LPS-induced cells and treated with 10^5 μ g/mL water extract).

Cell viability assay was carried out following the method from Zdařilová *et al.* (2009) [25] with modification. The cells were induced with LPS for 4 h. The LPS-induced cells were treated with R-38™ water extract for 24 h in 96-well plates. Then, the cells were washed with PBS twice and 20 μ L of MTT solution (5 mg/1 mL PBS) was added to each well. The plate was enclosed with aluminum foil and further incubated for 3.5 h. The MTT solution was discarded

from wells and 200 μL of non-sterile DMSO were added to dissolve the formazan produced in the cells. The plate reader (Model ELX 808, Bio-Tek, Winooski, VT) was used to measure the optical density of color formations of each sample at 570 nm and a reference wavelength at 630 nm. The LPS-induced cells, cultured separately in other 96-well plates, were used to measure IL-6 cytokines and TNF- α using commercially available Human ELISA kits based on the manufacturer's protocol. The intensity of the color was measured at 450 nm.

2.6 Quantification of Xanthine Oxidase and Cyclooxygenase-2 Inhibitory Activities

The xanthine oxidase (XO) inhibitory bioassay procedure was based on Li *et al.* (2011) [26]. The assay mixture was prepared by mixing 50 μL of test solution (samples or solvents), 35 μL of phosphate buffer (70 mM, pH = 7.5), and 30 μL of XO solution (0.01 U/mL in phosphate buffer). The assay mixture was incubated at 25 $^{\circ}\text{C}$ for 15 min. About 60 μL of xanthine solution (150 μM in the same buffer) was added into the assay mixture to initiate the reaction and further incubated for 30 min at 25 $^{\circ}\text{C}$. The reaction was stopped by adding 25 μL of 1 N hydrochloric acid. All assay was measured by spectrophotometer at a wavelength of 290 nm. Allopurinol was used as the positive control. Sample blank and negative control were prepared similarly, but the enzyme solution was added into the assay mixture after the addition of 1 N hydrochloric acid. The crude extracts were initially dissolved in DMSO, followed by serial dilutions with the buffer. The final concentration of DMSO for each extract was 0.5% v/v.

The cyclooxygenase-2 (COX-2) inhibitory bioassay method was conducted according to the COX-2 screening kit (BioVision K547-100, Milpitas, CA). A 100 μL of unknown concentration of celecoxib was provided in the kit as a positive control. The crude extracts were initially dissolved in DMSO, followed by serial dilutions with the COX assay buffer. The final concentration of DMSO for each test extract was 10% v/v. The prepared extracts were pipetted into each well followed by the addition of the master mixture. Lastly, 10 μL of arachidonic acid was added into each well and the reaction was initiated. A fluorescence microplate reader (Promega GloMax Multi Detection System, Wisconsin, WI) was used to read the plate with an emission wavelength of 587 nm and an excitation wavelength of 535 nm. The 96-well plate was measured kinetically at 25 $^{\circ}\text{C}$ for 5-10 min.

2.7 Experimental Animals

All experimental protocols were approved by the Ethics Committee and Research Management and Innovation Centre (Ref. No.: UMT/JKEPT/2018/20) of Universiti Malaysia Terengganu (UMT). The female Sprague-Dawley (SD) rats (5-7 weeks old, 180-200 g)

were acclimatized for 7 days before experimentation under standard laboratory conditions (24 $^{\circ}\text{C} \pm 3$ $^{\circ}\text{C}$ and 12 h light and dark cycle) with pellet feed and filtered water *ad libitum*.

2.8 Acute Oral Toxicity Study

The study was done according to the Fixed-dose procedure (FDP) from the Organization for Economic Co-operation and Development (OECD) 420 guideline [27]. Fifteen female SD rats were randomly assigned into the control group, treatment I, and treatment II groups. The R-38TM water extract was dissolved in 2 mL of distilled water and administered to rats by gavage single oral dose of 300 mg/kg body weight (bw) (treatment I) and 2000 mg/kg bw (treatment II) while the control group received only distilled water in the same volume. The general observations and mortality were observed daily for 14 days. At the end of the experiment, all rats were fasted overnight, weighed, anesthetized for sample blood collection, and sacrificed for necropsy.

2.9 Subacute Oral Toxicity Study

The study was conducted following the 28-day repeated dose from the OECD 407 (2008) guidelines [28]. A total of 30 female SD rats were divided into four groups of 5 female rats (control group, treatment I, treatment II, and treatment III). Additional rats ($n = 5$) were added to the control and treatment III groups as satellite groups for the recovery period. The R-38TM water extract was dissolved in 2 mL of distilled water and administered to rats in treatment groups at a daily oral dose of 300 mg/kg bw (treatment I), 1000 mg/kg bw (treatment II) and 2000 mg/kg bw (treatment III), while the control group received distilled water in the same volume for 28 days. The animals were fed orally, preferably, at the same time daily. The dose of 2000 mg/kg bw per day was selected as the maximum dose based on the acute toxicity investigation where the dose was well tolerated with no toxicity events or mortality. All rats were observed daily for general observations, clinical signs, and mortality until the end of the experiment. The satellite groups were kept for another 14 days in the recovery period to observe delayed occurrence of or recovery from toxic effects. At the end of the experiment, all rats were fasted overnight, weighed, anesthetized for blood samples collection, and sacrificed for necropsy.

2.10 Body and Organ Weight Analysis

The body weight of all animals in the acute oral toxicity study was measured on day 0 and day 15 (day of necropsy). Whereas the body weights of all animals in the subacute oral toxicity study were recorded once a week during the experiment and recovery period until the day of necropsy. During the scheduled necropsy, all animals were sacrificed, and the liver and kidneys of each rat were isolated,

washed with 0.9% normal saline, weighed, and fixed in 10% neutral buffered formalin for histopathological examination. The absolute organ weights were measured using electrical weighing balance and relative organ weights (organ-to-body weight ratio) were calculated from the rat's body weight on the day of necropsy.

2.11 Haematological and Biochemical Analysis

At the end of the experiment, all animals were anesthetized with a KTX mixture. The blood samples were collected via cardiac puncture. All blood samples were analyzed for hematological and biochemical analysis.

2.12 Histopathological Examination

The extracted liver and kidneys were washed with 0.9% normal saline and fixed in a 10% neutral buffered formalin solution on the day of scheduled necropsy. Briefly, the fixed liver and kidneys were sliced and placed in cassette holders and dehydrated in 50% ethanol, 70% ethanol, 95% ethanol, 100% ethanol and 100% toluene (overnight) gradually. The dried tissues were embedded in paraffin wax and sectioned at approximately 5–6 μm on a microtome. The tissue sections were prepared on glass slides and dried overnight before staining. The slides were stained with haematoxylin and eosin (H&E) dye solution. The slides were then affixed to the coverslip using DPX mountant. Slides of liver and kidney were examined under a light microscope (Leica DM2500, Wetzlar, Germany) for microscopic examination.

2.13 HPLC analysis of Rosmarinic Acid

High-performance liquid chromatography (HPLC) was performed for the quantification of rosmarinic acid in the R-38™ water extract. The standard rosmarinic acid was used as a calibration standard. Approximately 2.0 mg of R-38™ water extract was dissolved in 1 mL of 50% methanol (v/v; methanol in water) and vortexed for 5 min. The standard stock solution of rosmarinic acid was prepared by dissolving 2.0 mg in 1 mL of 50% methanol and vortexed for 5 min [29]. Five working standard solutions with a concentration of 20, 50, 100, 200, and 500 parts per million (ppm) were prepared from the stock solution. The instrument was calibrated and qualified before the analysis. The HPLC analysis was conducted using a Shimadzu Prominence system (Shimadzu Corporation, Japan) with an Agilent Zorbax Eclipse XDG-C18 column (4.6 x 150 mm, 5 μm pore size). The system control and data analysis were performed using Shimadzu laboratory solution system software. A mobile phase of acetonitrile (12 %) and ionized distilled water (88 %) was used for the analysis at 340 nm. The flow rate was 1 mL/min and maintained at 35 °C. The total sample injection volume was 20 μL and the total run time was 8 min. A

calibration curve of the standard was plotted for concentrations in the range of 20–500 ppm against the peak area.

2.14 Statistical Analysis

All data were expressed as mean and standard deviation (mean \pm SD). The homogeneity of variance test was conducted on all values. The comparison between treatment and control groups was performed by one-way analysis of variance (ANOVA) and the significant differences between individual groups were compared using post hoc Tukey's test with IBM SPSS Statistics 22 (IBM, Armonk, NY). The graphs were constructed using GraphPad Prism 5.0 (GraphPad Prism, San Diego, CA). The statistical level of significance was set at a $p < 0.05$.

3.0 RESULTS AND DISCUSSION

The treatment approach for gout, arthritis and other inflammatory-related diseases always involves the prescription of anti-inflammatory agents to alleviate the symptoms [30]. The ingredients of R-38™ are scientifically known to possess anti-inflammatory properties and have been mostly used in the treatment of arthritic conditions as well as a supplement for general health. The rosmarinic acid in the *O. stamineus* possessed anti-inflammatory activity and was responsible for managing arthritis-related conditions. Anti-inflammatory compounds are commonly detected in the HPLC analysis within the range of 200 to 350 nm [31]. In the HPLC analysis of R-38™ water extract, rosmarinic acid was identified at 1.15 min, at the wavelength of 340 nm (Figure 1). The retention time of rosmarinic acid in the water extract of R-38™ was comparable to the standard compound. The calibration plot was linear over the evaluated concentration range with the correlation coefficient (R^2) of rosmarinic acid compound was 0.9998. The concentration of rosmarinic acid in water extract of R-38™ from three replicates injections was $60.94 \pm 0.43 \mu\text{g/mL}$ or 3.61 % w/w.

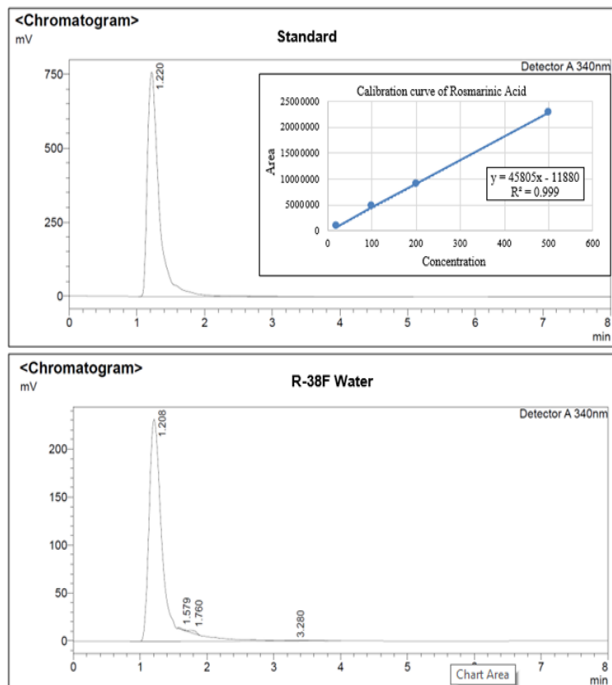


Figure 1 HPLC analysis of rosmarinic acid for standard and R-38™ water extract at 340 nm. The calibration plot of rosmarinic acid shows linear relationship with the correlation coefficient (R^2) of 0.9998

The activity of anti-inflammatory involved suppressing the pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β [32]. In this study, the anti-inflammatory activity of R-38™ water extract was evaluated from its inhibitory effect on the production of IL-6 and TNF- α . Lipopolysaccharide (LPS), an irritating agent isolated from the outer membrane of bacteria [25], was used to induce the inflammatory signals in the HSF1184 cell lines. The inflammatory effect from LPS-stimulated macrophages is a reliable marker of inflammation [31]. MTT assay was performed to determine the cytotoxic effect in normal fibroblast cells (HSF1184) against R-38™ water extract. As shown in Figure 2, the water extract of R-38™ exhibited no cytotoxic effect on the HSF1184 cells. Instead, the normal fibroblast cells have proliferated and showed no inhibitory effect at higher concentrations of 10^4 and 10^5 $\mu\text{g}/\text{mL}$. There was also a significant increase ($p < 0.05$) in the cell proliferation at 10^5 $\mu\text{g}/\text{mL}$ as compared to the control. The two highest concentrations of R-38™ water extract were then used in the anti-inflammatory activities. The R-38™ water extract significantly decreased ($p < 0.05$) the production of the IL-6 and TNF- α in the LPS-induced fibroblast cells in a dose-dependent manner (Figure 3). The production of IL-6 and TNF- α in the LPS-induced HSF1184 were suppressed by the R-38™ water extract suggesting a potential anti-inflammatory effect. As shown in Figure 4, the R-38™ water extract at 10^5 $\mu\text{g}/\text{mL}$ has significantly increased ($p < 0.05$) the survival rate of inflamed fibroblast cells as compared

to the normal fibroblast cells. Therefore, the study suggested that the water extract promotes cell growth and increases the LPS-induced HSF1184 cells survival rate by more than 50%.

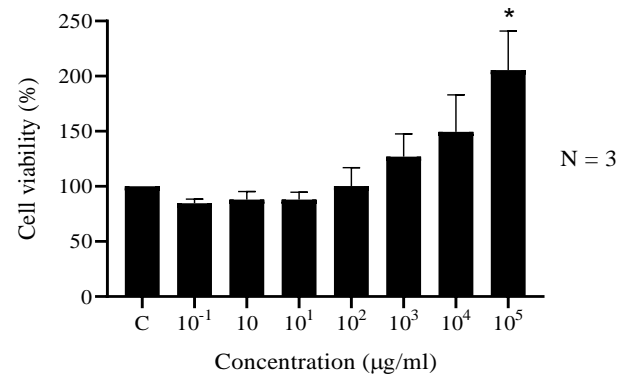


Figure 2 The effects of R-38™ water extract on cell viability of normal HSF1184 cell line using MTT assay. * $p < 0.05$ vs control group

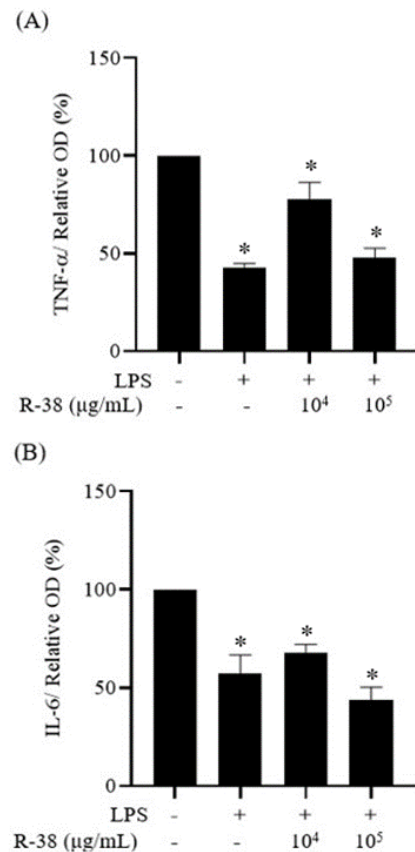


Figure 3 The effect of R-38™ water extract on the expression of inflammatory mediators in LPS-induced HSF1184 cell line. (A) The percentage of TNF- α production. (B) The percentage of IL-6 production. * $p < 0.05$ vs control group

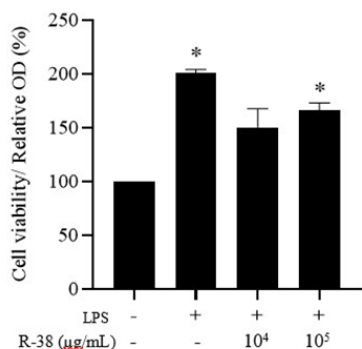


Figure 4 The effect of R-38TM water extract on cell viability of LPS-induced HSF1184 cell line. * $p < 0.05$ vs control group

Many diseases arise from the deficiency or excess of a specific metabolite in the body. Xanthine oxidase (XO) is an enzyme to catalyze hypoxanthine and xanthine into uric acids [33]. Excessive serum uric acid in the body, also known as hyperuricemia, may result in monosodium urate crystals deposition in joints and kidneys, which subsequently trigger the occurrence of acute inflammation. Gout is a common metabolic disorder closely related to hyperuricemia. There are many modern medicines available for gout such as XO inhibitors (allopurinol, febuxostat), uricosuric drugs (probenecid), cyclooxygenase-2 (COX-2) inhibitors, and non-steroidal anti-inflammatory drugs (NSAIDs). Despite their therapeutic effect, they also cause undesirable side effects to the body [34]. For example, allopurinol is very effective to stop the formation of uric acid via inhibition of the XO activity. However, the body metabolism in urate production will become abnormal in the absence of XO. Thus, the present study investigated the effect of R-38TM water extract in the inhibition of XO activity. The results showed that the extract moderately inhibited XO activity as compared to allopurinol (Table 1), which fulfilled the body requirement in maintaining the XO activity.

COX-2 and prostaglandins are associated with inflammatory diseases including arthritis as they are the mediators responsible for the progression of an inflammatory response [35]. Selective COX-2 inhibitor agents such as celecoxib and NSAIDs (e.g., indomethacin and diclofenac) are very effective drugs to inhibit COX-2 activity as well as produce anti-inflammatory, antipyretic, and analgesic activities [36]. Nevertheless, body functionality will be affected if COX-2 is lacking. Prostacyclin (PGI₂) also known as prostaglandin helps to relax the lining of the blood vessel and suppress platelet activation. The decrease in the amount of PGI₂ may increase the risk of cardiovascular incidence due to coagulation of platelets and abnormal clotting [37]. The present study investigated the inhibitory effect of R-38TM water extract on the COX-2 activity. The results found that R-38TM water extract showed a moderate (34.41%) COX-2 inhibition effect at 100 µg/mL (Table

1). Hence, based on the findings from both XO and COX2 enzymes inhibition assay, it is suggested that the anti-hyperuricemic mechanism of R-38TM may involve in the inhibition of XO and the prostaglandin pathway.

Table 1 *In vitro* xanthine oxidase (XO) and cyclooxygenase-2 (COX-2) inhibitory activity of R-38TM water extract

| Sample group (µg/ml) | Percentage of XO inhibition (%) | | | | | IC ₅₀ (µg/ml) |
|------------------------------------|---------------------------------|------------------|------------------|------------------|------------------|--------------------------|
| | 0.01 | 0.1 | 1 | 10 | 100 | |
| R-38 TM water extract | 27.9 8 ± 3.31 | 42.4 3 ± 1.75 | 36.4 4 ± 8.99 | 44.6 5 ± 4.44 | 56.2 3 ± 1.23 | 27.22 |
| Allopurinol (positive control) | 52.1 4 ± 4.56 | 66.1 9 ± 3.55 | 78.4 2 ± 4.07 | 81.0 0 ± 9.85 | 90.6 0 ± 0.60 | <0.01 |
| Percentage of COX-2 inhibition (%) | | | | | | |
| R-38 TM water extract | 36.1 8 ± 0.49 | 40.4 6 ± 1.45 | 37.1 2 ± 3.45 | 40.1 5 ± 1.04 | 34.4 1 ± 1.81 | - |

Unknown concentration of celecoxib was provided in the kit as a positive control with % inhibition of 79.18 ± 2.15

Values are expressed as mean ± Standard deviation.

R-38TM is an innovative polyherbal medicine developed through the Malay wisdom in traditional herbal medicine. This formulation was traditionally used for treating health conditions related to inflammation of joints, bones, and nerves. Though many previous studies have reported on the safety of each herb, there is no available research on the safety of this polyherbal formulation. In the present study, *in vitro* and *in vivo* studies on the R-38TM water extract were carried out to determine the toxicity potential of R-38TM. The *in vitro* test was conducted to determine the cytotoxicity potential of R-38TM against the HSF1184 cell line. According to the National Institutes of Health [38], the LD₅₀ (50% lethal dose) value for safety study on laboratory animals (i.e., rodents) can be estimated from the IC₅₀ value obtained from *in vitro* cytotoxicity test. This approach provides a starting dose closer to the actual LD₅₀ value for *in vivo* acute oral toxicity study. From this study, the water extract of R-38TM has no LD₅₀ value as there was no inhibitory effect seen in the cytotoxicity assay. Thus, the selected starting dose for acute oral toxicity study was 300 mg/kg as suggested by the OECD 420 guideline. The starting dose of this guideline is within the safety range of R-38TM based on the results obtained.

The *in vivo* safety study of the R-38TM water extract was investigated through acute and subacute oral toxicity study on SD female rats following the OECD guidelines. The OECD guideline suggested a pre-determined fixed dose, and from the outcome, a test substance is ranked and classified according to the Globally Harmonized System (GHS) [39]. The acute oral toxicity study was carried out on 15 female SD

rats tested with a single oral dose of R-38™ water extract at 300 mg/kg and 2000 mg/kg. The findings revealed that the water extract did not exhibit any toxicity effects, sudden death, or mortality. The body weight changes of all rats (Table S1) showed no significant difference ($p > 0.05$) as compared to the control group, which indicated that the treatment of R-38™ water extract did not obstruct the growth of rats even up to the highest dose of 2000 mg/kg. There was also no significant difference ($p > 0.05$) in the absolute and relative organ weight (Table S2).

The hematological (Table S3) and biochemical analysis (Table S4) on treatment I (300 mg/kg) had shown no treatment-related toxicity effects, but treatment II (2000 mg/kg) showed a significant increase ($p < 0.05$) in WBC counts and decrease in albumin level. Regardless, the common markers of toxicity which were the liver profile (ALT, AST, and ALP) and kidney profile (creatinine and BUN) showed no significant ($p > 0.05$) alterations. It is most likely that the significant differences were caused by within-group variation. The histopathological evaluation also showed no treatment-related morphological alterations in the liver and kidneys of all the treatments after a single dose of R-38™ water extract (Figure S1). It caused no significant ($p > 0.05$) organ damage and occurrence of macroscopic and microscopic changes. The LD₅₀ of R-38™ water extract was estimated to be more than 2000 mg/kg

by oral administration. Herbal medicines with LD₅₀ values greater than 2000 mg/kg were considered safe for acute exposure [40–41].

The study of subacute oral toxicity was conducted on four groups of rats treated with a repeated daily oral dose of 300 mg/kg, 1000 mg/kg, and 2000 mg/kg of R-38™ water extract and vehicle for 28 days. The findings of this study revealed that the R-38™ water extract was well tolerated and did not obstruct the growth of all the treated rats even up to the highest dose of 2000 mg/kg (Table S5). The absolute and relative organ weight of all treated rats were found to be statistically insignificant ($p > 0.05$) with respect to the control group (Table S6). All hematological parameters of the three treatment groups were well within the normal limits as compared to the control group (Table 2). The biochemical analysis of all treated groups also showed no significant differences as compared to the control group (Table 3). The histopathological analysis on the liver and kidney of the three treatment groups revealed no treatment-related abnormalities in the cell morphology (Figure 5 and Figure 6). The satellite treatment group (2000 mg/kg) was maintained for another 14 days in the recovery period to observe potential recovery or delayed toxicity events. Consequently, the findings revealed no mortality or occurrence of any delayed toxicity events in all assessments.

Table 2 Haematological values of SD rats orally administrated with R-38™ water extract in the 28-day repeated oral dose study and recovery period.

| Parameter | Dose (mg/kg) | | | | | |
|-------------------------------|----------------|-----------------|---------------|----------------|----------------|----------------|
| | Control | 300 | 1000 | 2000 | Control RP | 2000 RP |
| WBC ($\times 10^9/L$) | 7.9 ± 3.78 | 7.9 ± 2.01 | 9.7 ± 2.63 | 10.6 ± 2.22 | 8.8 ± 3.25 | 7.9 ± 3.86 |
| RBC ($\times 10^{12}/L$) | 6.5 ± 0.84 | 6.6 ± 0.16 | 6.5 ± 0.39 | 6.7 ± 0.84 | 6.4 ± 0.51 | 6.7 ± 0.52 |
| Haemoglobin (g/dL) | 12.8 ± 1.62 | 13.8 ± 0.35 | 13.6 ± 0.50 | 12.7 ± 1.01 | 12.9 ± 1.17 | 12.8 ± 0.38 |
| Haematocrit (%) | 37.75 ± 4.99 | 38.25 ± 0.96 | 37.25 ± 1.71 | 36.60 ± 3.85 | 37.00 ± 2.55 | 37.40 ± 1.95 |
| MCV (fl) | 58.0 ± 0.82 | 58.3 ± 2.06 | 57.5 ± 1.00 | 54.8 ± 2.86 | 58.4 ± 2.07 | 56.4 ± 2.19 |
| MCH (pg) | 19.8 ± 1.50 | 21.0 ± 0.82 | 21.0 ± 0.00 | 19.2 ± 1.64 | 20.4 ± 1.14 | 19.2 ± 1.30 |
| MCHC (g/L) | 340.0 ± 24.49 | 362.5 ± 5.00 | 365.0 ± 5.77 | 348.0 ± 17.89 | 348.0 ± 13.04 | 342.0 ± 13.04 |
| Platelets ($\times 10^9/L$) | 700.0 ± 335.83 | 1018.0 ± 196.96 | 762.5 ± 74.84 | 614.0 ± 177.65 | 884.8 ± 136.64 | 838.0 ± 111.59 |
| Neutrophils (%) | 33.3 ± 9.91 | 30.3 ± 9.18 | 28.5 ± 13.08 | 27.4 ± 18.53 | 29.4 ± 8.38 | 43.4 ± 17.30 |
| Lymphocytes (%) | 60.8 ± 10.72 | 66.0 ± 9.06 | 67.0 ± 11.34 | 69.2 ± 20.97 | 62.6 ± 6.50 | 50.6 ± 18.93 |
| Eosinophils (%) | 3.3 ± 2.36 | 1.5 ± 1.73 | 1.8 ± 1.50 | 1.0 ± 1.73 | 4.6 ± 2.79 | 3.4 ± 2.30 |
| Monocytes (%) | 2.5 ± 0.58 | 2.3 ± 1.71 | 2.8 ± 2.22 | 2.4 ± 1.67 | 3.2 ± 2.49 | 2.6 ± 1.14 |
| Basophils (%) | 0.0 ± 0.00 | 0.0 ± 0.00 | 0.0 ± 0.00 | 0.0 ± 0.00 | 0.0 ± 0.00 | 0.0 ± 0.00 |

Values are expressed as mean ± Standard deviation ($n = 5$; for each group). RP: Recovery period; RBC: red blood cell count; WBC: white blood cell count; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration

Table 3 Serum biochemical parameters of SD rats orally administrated with R-38™ water extract in the 28-day repeated oral dose study and recovery period

| Parameter | Dose (mg/kg) | | | | | |
|----------------------------|----------------|---------------|---------------|---------------|---------------|----------------|
| | Control | 300 | 1000 | 2000 | Control RP | 2000 RP |
| AST (U/L) | 207.6 ± 130.78 | 153.8 ± 23.21 | 215.6 ± 53.97 | 132.8 ± 18.28 | 204.0 ± 76.72 | 149.0 ± 30.86 |
| ALT (U/L) | 74.2 ± 44.86 | 70.4 ± 7.70 | 65.0 ± 19.20 | 42.8 ± 7.16 | 75.6 ± 11.06 | 60.8 ± 9.65 |
| ALP (U/L) | 197.6 ± 55.30 | 212.2 ± 89.91 | 157.6 ± 32.34 | 132.8 ± 55.36 | 166.8 ± 49.33 | 130.4 ± 116.67 |
| Urea (mmol/L) | 8.2 ± 1.30 | 8.4 ± 0.78 | 8.0 ± 0.38 | 7.5 ± 1.29 | 8.3 ± 0.99 | 7.9 ± 0.72 |
| Creatinine (µmol/L) | 56.1 ± 4.1 | 62.8 ± 4.11 | 62.0 ± 3.15 | 59.3 ± 5.30 | 59.0 ± 7.26 | 59.7 ± 3.20 |
| BUN (mmol/L) | 8.2 ± 1.30 | 8.4 ± 0.78 | 8.0 ± 0.38 | 7.5 ± 1.29 | 8.3 ± 0.99 | 7.9 ± 0.72 |
| Uric acid (µmol/L) | 125.6 ± 17.43 | 145.9 ± 47.95 | 130.4 ± 42.79 | 141.1 ± 27.92 | 138.7 ± 40.87 | 120.8 ± 16.05 |
| Total protein (g/L) | 77.4 ± 3.78 | 79.2 ± 3.63 | 69.6 ± 3.21 | 70.4 ± 4.98 | 79.4 ± 6.02 | 80.2 ± 4.15 |
| Albumin (g/L) | 30.0 ± 4.53 | 33.0 ± 2.45 | 27.2 ± 1.10 | 29.8 ± 2.17 | 34.4 ± 4.34 | 33.2 ± 1.64 |
| Globulin (g/L) | 47.4 ± 7.20 | 46.2 ± 3.35 | 42.4 ± 2.30 | 40.6 ± 4.04 | 45.0 ± 3.32 | 47.0 ± 2.65 |
| Total cholesterol (mmol/L) | 1.6 ± 0.29 | 1.8 ± 0.21 | 1.5 ± 0.34 | 1.5 ± 0.18 | 2.0 ± 0.51 | 2.2 ± 0.47 |
| Triglycerides (mmol/L) | 1.4 ± 0.78 | 1.5 ± 0.36 | 1.6 ± 0.44 | 1.3 ± 0.65 | 1.8 ± 0.41 | 1.1 ± 0.43 |
| Glucose (mmol/L) | 11.4 ± 2.85 | 14.1 ± 3.39 | 14.9 ± 3.41 | 12.2 ± 4.35 | 10.1 ± 2.43 | 7.9 ± 1.55 |

Values are expressed as mean ± Standard deviation (n = 5; for each group), $p < 0.05$ using one-way ANOVA followed by Tukey's multiple comparison test; * $p < 0.05$ vs control group. RP: Recovery period; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; BUN: blood urea nitrogen

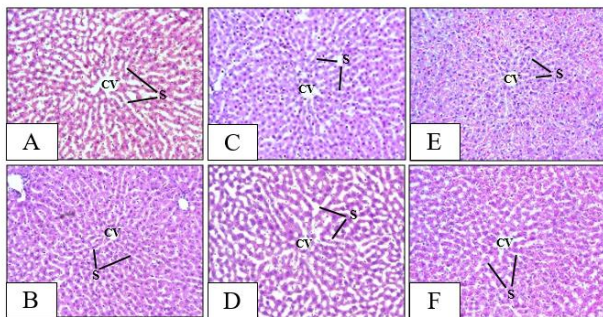


Figure 5 Photomicrographs of paraffin section of liver stained with H&E in subacute oral toxicity study (Magnification 200×). (A) Liver treated with 300 mg/kg bw; (B) Liver treated with 1000 mg/kg bw; (C) Liver treated with 2000 mg/kg bw; (D) Liver of satellite rat treated with 2000 mg/kg bw; (E) Liver of rat in control group; (F) Liver in satellite control group. CV – central vein; S – sinusoid

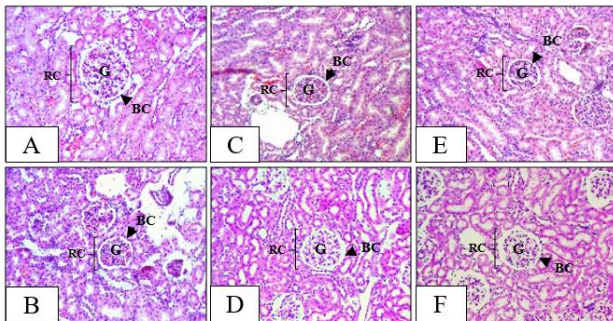


Figure 6 Photomicrographs of paraffin section of the kidney stained with H&E in subacute oral toxicity study (Magnification 200×). (A) Kidney treated with 300mg/kg bw; (B) Kidney treated with 1000mg/kg bw; (C) Kidney treated with 2000mg/kg bw; (D) Kidney of satellite rat treated with 2000mg/kg bw; (E) Kidney in control group; (F) Kidney in satellite control group. RC – Renal corpuscle; BC- Bowman's capsule; G- glomerulus

4.0 CONCLUSION

In sum, the in vitro study revealed that R-38™ water extract inhibited the production of IL-6 and TNF- α in the LPS-induced fibroblast cells. Furthermore, the water extract might potentially treat the arthritis related-conditions by its ability to moderately inhibit the XO and COX-2 activities. The R-38™ water extract also caused no cytotoxic effect on fibroblast cells up to the highest dose of 2000 mg/kg, however, its IC₅₀ value could not be determined. The studies of acute and subacute oral toxicity of R-38™ water extract did not exhibit any treatment-related toxicity at all doses. The LD₅₀ value of the R-38™ water extract was estimated to be more than 2000 mg/kg. The R-38™ showed potential anti-inflammatory activity and can be considered as a promising therapeutic medicine against pro-inflammatory diseases including arthritis. The present findings could serve as preliminary data for a long-term toxicity study, which is necessary to further evaluate the efficacy and safety of R-38™.

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Appendix

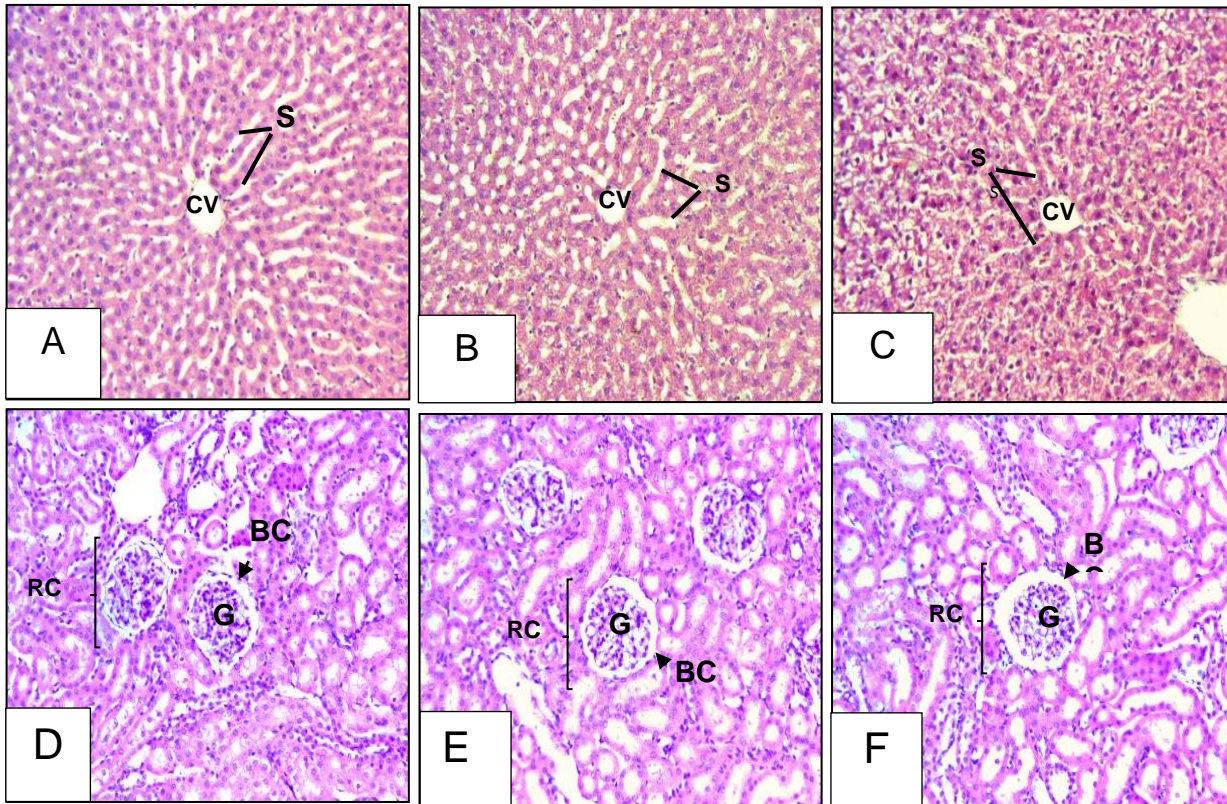


Figure S1 Photomicrographs of paraffin section of the liver and kidney stained with Haematoxylin & Eosin (H&E) solution in acute oral toxicity study (Magnification 200×). (A) Normal liver structure of control group; (B) Liver treated with 300 mg/kg bw; (C) Liver treated with 2000mg/kg bw. (D) Normal kidney structure of control group; (E) Kidney treated with 300 mg/kg bw; (F) Kidney treated with 2000 mg/kg bw. CV: Central vein; BC: Bowman's capsule; S: Sinusoid; G: Glomerulus; RC: Renal corpuscle

Table S1 Body weight of CD rats orally administered with R-38™ water extract in the acute oral toxicity study

| Group | Dose level | Body Weight pre-treatment (g); (n = 5) | Body Weight 14 days post-treatment (g); (n = 5) |
|--------------|----------------------|---|--|
| Control | 2 ml distilled water | 194.0 ± 8.94 | 210.0 ± 10.00 |
| Treatment I | 300 mg/kg bw | 192.0 ± 17.89 | 208.0 ± 21.68 |
| Treatment II | 2000 mg/kg bw | 190.0 ± 14.14 | 222.0 ± 27.75 |

Values are expressed as mean ± Standard deviation (n = 5; for each group); n = number of rats.

Table S2 Absolute and relative organ weight of CD rats following a single dose oral administration with R-38™ water extract

| Dose (mg/kg bw) | Control | | 300 | | 2000 | |
|--------------------|-------------------|----------------|----------------|----------------|----------------|-------------------|
| | Absolute (g) | Relative (%) | Absolute (g) | Relative (%) | Absolute (g) | Relative (%) |
| Liver | 7.792 ± 0.6906 | 3.723 ± 0.4309 | 7.930 ± 0.9713 | 3.810 ± 0.2006 | 8.126 ± 0.9453 | 3.609 ± 0.6239 |
| Kidney | 1.436 ± 0.1106 | 0.687 ± 0.0810 | 1.376 ± 0.2215 | 0.662 ± 0.0837 | 1.578 ± 0.2146 | 0.714 ± 0.0846 |

Values are expressed in mean ± Standard deviation (n = 5; for each group).

Table S3 Haematological parameters of CD rats after a single dose of R-38™ water extract

| Parameter | Dose (mg/kg bw) | | |
|---------------------------------|-----------------|-----------------|----------------|
| | Control | 300 | 2000 |
| WBC (x10 ⁹ /L) | 4.83 ± 1.25 | 4.90 ± 1.81 | 9.10 ± 3.81* |
| RBC (x10 ¹² /L) | 6.65 ± 0.13 | 6.60 ± 0.52 | 6.66 ± 0.30 |
| Haemoglobin (g/dL) | 14.48 ± 0.46 | 13.83 ± 0.96 | 13.30 ± 0.66 |
| Haematocrit (%) | 39.25 ± 1.71 | 38.40 ± 2.61 | 37.60 ± 0.89 |
| MCV (fl) | 59.25 ± 0.96 | 58.40 ± 0.89 | 56.40 ± 2.07 |
| MCH (pg) | 21.75 ± 0.96 | 21.20 ± 0.45 | 19.80 ± 1.64 |
| MCHC (g/L) | 370.0 ± 14.14 | 360.0 ± 0.00 | 356.0 ± 20.74 |
| Platelets (x10 ⁹ /L) | 771.25 ± 86.15 | 659.00 ± 342.94 | 906.80 ± 68.79 |
| Neutrophils (%) | 44.0 ± 10.10 | 34.8 ± 7.19 | 34.8 ± 5.76 |
| Lymphocytes (%) | 53.3 ± 9.91 | 63.4 ± 8.79 | 60.6 ± 4.39 |
| Eosinophils (%) | 0.5 ± 1.00 | 0.2 ± 0.45 | 1.0 ± 1.00 |
| Monocytes (%) | 2.3 ± 0.96 | 1.6 ± 1.34 | 3.6 ± 1.67 |
| Basophils (%) | 0.0 ± 0.00 | 0.0 ± 0.00 | 0.0 ± 0.00 |

Values are expressed as mean ± Standard deviation (*n* = 5; for each group). *p* < 0.05 using one-way ANOVA followed by Tukey's multiple comparison test; * *p* < 0.05 vs control group. RBC: red blood cell count; WBC: white blood cell count; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration.

Table S4 Serum Biochemical parameters of CD rats after a single dose of R-38™ water extract

| Parameter | Dose (mg/kg bw) | | |
|----------------------------|-----------------|-----------------|----------------|
| | Control | 300 | 2000 |
| AST (U/L) | 175.40 ± 66.53 | 145.60 ± 43.60 | 180.80 ± 31.56 |
| ALT (U/L) | 69.40 ± 24.42 | 76.80 ± 14.46 | 71.40 ± 19.01 |
| ALP (U/L) | 274.60 ± 107.42 | 259.80 ± 127.09 | 339.40 ± 96.93 |
| Creatinine (µmol/L) | 61.78 ± 2.88 | 60.54 ± 3.26 | 57.55 ± 5.62 |
| Urea (mmol/L) | 8.60 ± 1.85 | 7.89 ± 0.61 | 8.53 ± 1.28 |
| BUN (mmol/L) | 8.60 ± 1.85 | 7.89 ± 0.61 | 8.53 ± 1.28 |
| Uric acid (µmol/L) | 131.56 ± 19.72 | 160.26 ± 107.84 | 137.54 ± 11.19 |
| Total protein (g/L) | 78.6 ± 2.51 | 75.8 ± 6.91 | 75.2 ± 8.76 |
| Albumin (g/L) | 33.4 ± 1.14 | 32.6 ± 2.61 | 27.6 ± 4.39 * |
| Globulin (g/L) | 45.2 ± 1.92 | 43.2 ± 4.55 | 47.6 ± 12.10 |
| Total cholesterol (mmol/L) | 1.86 ± 0.065 | 1.92 ± 0.096 | 1.58 ± 0.348 |
| Triglyceride (mmol/L) | 1.626 ± 1.2509 | 1.694 ± 0.8123 | 1.888 ± 0.4611 |
| Glucose (mmol/L) | 12.73 ± 1.347 | 13.38 ± 4.738 | 10.98 ± 2.949 |

Values are expressed as mean ± Standard deviation (*n* = 5; for each group). *p* < 0.05 using one-way ANOVA followed by Tukey's multiple comparison test; * *p* < 0.05 vs control group. AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; BUN: blood urea nitrogen.

Table S5 Body weight of SD rats orally administrated with R-38™ water extract in the 28-day repeated oral dose study and recovery period (satellite groups)

| Group | Pre-treatment Weight | BW Week 1 | BW Week 2 | BW Week 3 | BW Week 4 | BW Week 5 | BW Week 6 |
|----------------------------------|----------------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Control | 189.0 ± 12.87 | 191.0 ± 15.24 | 193.0 ± 12.52 | 196.0 ± 13.50 | 196.0 ± 16.47 | 206.0 ± 17.13 | 210.0 ± 14.14 |
| 300 mg/kg bw (Treatment I) | 200.0 ± 20.00 | 204.0 ± 8.94 | 214.0 ± 8.94 | 220.0 ± 7.07 | 224.0 ± 11.40 | NIL | NIL |
| 1000 mg/kg bw (Treatment II) | 192.0 ± 17.89 | 186.0 ± 11.40 | 194.0 ± 11.40 | 202.0 ± 16.43 | 212.0 ± 21.68 | NIL | NIL |
| 2000 mg/kg bw (Treatment III) | 196.0 ± 17.76 | 201.0 ± 15.24 | 203.0 ± 13.37 | 207.0 ± 17.04 | 212.0 ± 18.74 | 210.0 ± 18.26 | 200.0 ± 0.00 |

Values are expressed as mean ± Standard deviation (n = 5; for each group); BW: Body weight.

Table S6 Absolute and relative organ weight of SD rats following the 28-days repeated dose oral administration of R-38™ water extract and recovery period

| Organ | Liver | | Kidney | |
|---------------|--------------|--------------|--------------|--------------|
| | Absolute (g) | Relative (%) | Absolute (g) | Relative (%) |
| Control | 7.73 ± 1.462 | 3.81 ± 0.496 | 1.54 ± 0.224 | 0.76 ± 0.074 |
| 300 mg/kg | 1.54 ± 0.224 | 0.76 ± 0.074 | 1.76 ± 0.191 | 0.78 ± 0.075 |
| 1000 mg/kg | 6.88 ± 1.200 | 3.23 ± 0.290 | 1.28 ± 0.115 | 0.61 ± 0.039 |
| 2000 mg/kg | 7.08 ± 1.462 | 3.34 ± 0.718 | 1.62 ± 0.181 | 0.76 ± 0.096 |
| Control RP | 7.22 ± 0.485 | 3.44 ± 0.083 | 1.57 ± 0.179 | 0.75 ± 0.064 |
| 2000 mg/kg RP | 6.36 ± 1.440 | 3.18 ± 0.720 | 1.63 ± 0.098 | 0.82 ± 0.049 |

Values are expressed as mean ± Standard deviation (n = 5; for each group); RP: Recovery period.