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Acute oral toxicity and anti-inflammatory evaluation of methanolic extract of *Rotula aquatica* roots in Wistar rats

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**ABSTRACT**

**Background:** The plant *Rotula aquatica* Lour. was traditionally well known due to its large number of pharmacological action and medicinal uses. The plant is a necessary component of many Ayurvedic drug preparations since historical times. It is widely used as a crucial ancient drug for kidney and bladder stones.  

**Objectives:** The main objective of the study was to evaluate the acute toxicity and anti-inflammatory efficacy of methanolic extract of *R. aquatica* Lour. in *in vivo* models.  

**Materials and methods:** The qualitative phytochemical analysis and invitro antioxidant activity of the roots of methanolic extract of *R. aquatica* Lour. (MERA) was evaluated. The acute toxicity effect of MERA was evaluated with two different doses (550, 2000 mg/kg body weight), were administrated orally to Wistar rats. The rats were observed for sign and symptoms of toxicity and mortality for 14 days. The parameters measured including relative organ weight, blood, biochemical and histopathological parameters of hepatic and renal toxicity. The anti-inflammatory effect of MERA was also evaluated in carrageenan and dextran-induced paw edema models.  

**Results:** The phytochemical evaluation of MERA shows the presence of secondary metabolites like alkaloids, flavonoids, phenolics and tannins, phytosterols, reducing sugars, proteins and terpenoids. The results of in-vitro antioxidant evaluation of MERA reveal its capability to scavenging free radical at a lower concentration. The MERA did not show any visible signs of toxicity up to the dose of 2000 mg/kg body weight. The results obtained from our carrageenan and dextran-induced paw edema model study also proved the anti-inflammatory effect of MERA in rat model.  

**Conclusion:** The result shows the potential of MERA as an anti-inflammatory drug to reduce the signs of inflammation devoid of any toxic effect.

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1. Introduction

Around the world numerous native communities possess knowledge, innovations and peculiar practices developed from expertise gained over centuries and tailored to the native culture and environment, such knowledge is categorized as traditional knowledge. The biodiversity rich areas are also an abode of various ethnic groups possessing a valuable reservoir of indigenous knowledge system (IKS) acquired and developed over a long period of time [1]. Within the indigenous system of medicine that prevalent in India, *R. aquatica* Lour. is a controversial drug and is a widely distributed shrub commonly found in the sandy soils and rocky beds of streams and rivers throughout India. The plant has been reported to be used for diabetes, treatment of piles, venereal disease, cancer and also exhibited anthelmintic activity [2], cardiotonic activity, antiinflammatory activity [2], antilithiatic activity etc. Despite the increasing number of reports on the medicinal benefits of the *R. aquatica* Lour., the in-vivo toxicological effect of the MERA has not reported so far. It is therefore deemed necessary to evaluate the acute toxicity of the MERA in a rat model. The toxicity study would serve as a very important baseline for further studies in developing this plant as a herbal medicine.

Inflammation is a major process involved in the pathophysiology of several diseases like arthritis and cardiovascular disease [3]. The anti-inflammatory drugs exert its anti-inflammatory
actions by inhibiting cyclooxygenase (COX) which exists as two isoforms: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) [4]. The COX-1 is constitutively expressed in most cells under physiological conditions and helps in maintaining gastrointestinal tract mucosal integrity, renal protection and normal homeostasis of the body [5]. COX-2, the form induced by pro-inflammatory agents such as tumor necrosis factor-alpha (TNF-α), lipopolysaccharide (LPS) and tumor-promoting factors are associated with inflammation [6]. Current treatment strategies are mainly associated with the usage of nonsteroidal anti-inflammatory drugs (NSAIDs) which are the known inhibitors of COX isoforms [7]. The usage of such kind of drugs causes serious side effects to human kind. For years, research has been focused on developing COX-2 inhibitors but recent investigation points out that COX-2 specific inhibitors are associated with adverse renal effects like sodium, potassium and water retention as well as decreases in renal function. The inhibition of COX-2 was associated with increase in production of leukotrienes (LT) by 5-lipoxygenase (5-LOX). The 5-LOX inhibitors possess protective effect but they lack inhibitor specificity, bioavailability and also leads to serious abnormalities [8]. Such complex side effects have limited COX and 5-LOX inhibitors for the long-term treatment of inflammatory disorders. Now a day, research has been focused on finding anti-inflammatory agents with selective pharmacology and less toxicity. From the above context, the study was designed to evaluate the anti-inflammatory effect of MERA in in vivo model.

2. Materials and methods

2.1. Chemicals

All the chemicals used were high-quality analytical grade reagents. Indomethacin (Merck, Bangalore, India) and Carrageenan (Sigma Chemicals, St. Louis, MO, USA) were procured from the respective companies and were used in the study.

2.2. Plant materials

*R. aquatica* Lour. roots were collected during March–April, 2016 from Meenachil river (Kalathukadavu area, 9.7° N, 76.78° E) in Kottayam district, Kerala, India. The plant material was authenticated by taxonomist Dr. Jomy Augustine, St. Thomas College, Palai, a voucher specimen (SBSBRL.22) is maintained in the institute.

2.3. Preparation of MERA

Plant root powder (40 g) was extracted with 400 mL of 99% methanol using a Soxhlet apparatus. The solvent was evaporated under reduced pressure at 50°C using a rotary evaporator to produce a yield of 4.28% of dry extract. The dried MERA was used for further analysis.

2.4. Phytochemical analysis

The qualitative phytochemical analysis of MERA was carried out by the following methods proposed by Evans et al. [9]. The total phenolic content was determined by spectrometry using Folin–Ciocalteu reagent assay [10]. Total flavonoid content was determined by a colorimetric assay using a method described by Zhishen et al. [11].

2.5. In-vitro antioxidant assay

DPPH assay, ABTS assay and Superoxide scavenging assay were carried out for the determination of antioxidant activity of MERA. DPPH free radical scavenging activity of MERA was determined by the method described by Sanchez-Moreno et al. [12]. For ABTS assay, the procedure followed the method of Arnao et al. [13]. Superoxide scavenging assay of MERA was done by Gulcin et al. [14].

2.6. Toxicity studies of MERA

The oral acute toxicity study of MERA was evaluated according to Organization for Economic Cooperation and Development (OECD) guideline 423 [15]. LD50 was determined by using Acute Oral Toxicity Statistical Program (Version: 1.0).

2.6.1. Animals

Adult female Wistar rats (weighing 150 ± 10 g) were purchased from small animal breeding station (SABS), Kerala Veterinary and Animal Sciences University, Mannuthy, Thrisur, Kerala used for this study. They were kept in a controlled environment for temperature (24–26°C), humidity (55–60%) and photoperiod (12:12 h light–dark cycle). A commercial laboratory balanced diet (Amrut Laboratory Animal Feeds, Maharashtra, India) and tap water were provided ad libitum. The animals received humane care, in compliance with the host institutional animal ethics guidelines. Experiments were conducted as per the guidelines of Institutional Animal Ethical Committee, School of Biosciences, Mahatma Gandhi University (Reg. No. MGLUSBS/IAEC/2016-B11042016/5) according to Government of India accepted principles for laboratory animals’ use and care.

2.6.2. Experimental design for acute toxicity studies

Animals were categorized into three different groups with 6 rats in each group. The group-I was treated as the control group (received distilled water only). Group II (MERA was administered orally at 550 mg/kg body weight) and group III (MERA was administered orally at 2000 mg/kg body weight) served as test groups. All the rats were fasted prior to oral gavage with MERA for 1–2 h. Individual body weights of animals were taken before dosing. Food or water was withheld for 2 h after drug treatment. The animals were closely monitored for initial 4 h after the administration of MERA and then daily for 14 days to record any signs of toxicity such as tremors, convulsions, salivation, hyperactivity, diarrhea, lethargy, sleep, coma and mortality [16,17]. At the end of the study, all the animals were sacrificed to analyze the effect of MERA on different organs of the rat.

2.6.3. Measurement of relative organ weight

After the experiment, organs like kidney, liver and heart were taken and organ weight (absolute organ weight in g) was recorded. The relative organ weight (ROW) of kidney, liver and heart were calculated as per the method described by Pichika et al. [18].

2.6.4. Estimation of hepatic and renal toxicity markers

Alkaline phosphatase (ALP), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), blood urea nitrogen (BUN), Creatinine activity in serum and protein concentration were assayed using commercial kits obtained from Span Diagnostics Limited, India, and the absorbance was read in a UV–Vis spectrophotometer (U-5100, Hitachi High Technologies, America, Inc.).

2.6.5. Histopathological study

Small blocks of tissues were taken from the organs (kidney, liver and heart) harvested from the rats and processed using an automated tissue processor. After processing, the tissues were sectioned to a thickness of 5 μm using a rotary microtome and dried overnight in an oven at 37°C. The sections were stained with hematoxylin and eosin (H&E) and examined microscopically for signs of toxicity.
2.7. Anti-inflammatory studies

2.7.1. Carrageenan-induced paw edema

Adult female Wistar rats (weighing 150 ± 10 g) were used for the study. An edema was induced on rat’s right hind paw by subplantar injection of 0.1 ml of 1% carrageenan in 0.9% saline. The experimental groups consisted of 30 rats divided into five groups — Group I: control (received saline only), Group II: carrageenan alone, Group III: carrageenan + indomethacin (3 mg/kg orally), Group IV: carrageenan + MERA-100 (100 mg/kg orally), Group V: carrageenan + MERA-200 (200 mg/kg orally). The MERA and reference drug were given 1 h before the injection of carrageenan. The volume of the right paw was measured using a paw edema meter (Marsap Pvt. Ltd., USA) before injection and 1st, 2nd, 3rd, 4th and 5th h after induction of inflammation. The results were obtained by measuring the volume difference before and after injection of the right paw. The swelling degree of paw and inhibition rate of edema was calculated as follows: % edema inhibition = (Vc – Vt) × 100/Vc; Vc and Vt are average edema volume of control and test, respectively [19].

2.7.2. Dextran-induced paw edema

The animals (Adult female Wistar rats) were treated in a manner similar to that of carrageenan-induced paw edema protocol, differing only in the administration of the inflammatory stimulus, which was induced by subplantar injection of 0.1 ml of 1.5% w/v dextran. The edema was measured as mentioned earlier [19] at hourly intervals for 5 h [20].

2.7.3. Histopathological analysis of paw tissue

The entire paw tissue sections (5 μm) fixed by immersion at room temperature in 10% formalin solution. For histopathological examinations, paraffin-embedded paw tissue sections were stained with hematoxylin–eosin (H&E) followed by examination and photographed under a light microscope for observation of structural abnormality. The severity of paw tissue inflammation was judged by two independent observers blinded to the experimental protocol.

2.8. Statistical analysis

The results were expressed as mean ± SD where each value represents a minimum of 6 rats (n = 6). The data for relative organ weights, hematology and serum biochemistry were tested for one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison tests in which the results were compared with that of control rats. The results were considered statistically significant at p ≤ 0.05 level. GraphPad Prism® version 5.03 for Windows (GraphPad Software, USA) was used for all statistical analysis.

3. Results

3.1. Phytochemical analysis of MERA

Qualitative detection test for important phytochemicals present in MERA revealed the presence of secondary metabolites like alkaloids, flavonoids, phenolics and tannins, phytosterols, reducing sugars, proteins and terpenoids. The qualitative phytochemical screening proved the presence of phenolic compounds as major principle components. Total phenolic content was found to be 51.27 ± 0.68 mg of gallic acid/g Dry weight of the sample. Total flavonoid content was found to be 16.59 ± 0.1 and expressed in mg of quercetin/g Dry weight of the sample.

3.2. In-vitro antioxidant effect of MERA

In-vitro antioxidant effect of MERA was examined using three different assays. The IC50 value of DPPH radical scavenging activity of MERA was 14.71 ± 0.03 μg/mL. The IC50 value of ABTS+ scavenging and superoxide scavenging activity of MERA were 12.82 ± 0.08; 36.02 ± 0.05 μg/mL respectively.

3.3. Acute toxicity study of MERA

Oral administration of MERA at a dose of 2000 mg/kg body weight did not produce any mortality or adverse effects during the 14 days’ period of study and also observed no abnormal clinical signs. The body weight and food consumption were also remaining unchanged, when compared to the untreated control group of animals.

3.3.1. Measurement of relative organ weight

There was no significant change (p > 0.05) in the weight of liver, kidney and heart of the treated and non-treated rats (Table 1). The relative organ weight of the rats of treatment, as well as control groups, also remained insignificant.

3.3.2. Estimation of hepatic and renal toxicity markers

The results of liver function markers (AST, ALT, ALP) used in the acute toxicity study (Table 1) showed no significant change (p > 0.05) between the control and MERA administered rats. Table 2 represents the results of renal function tests used to assess the kidney function status during the acute toxicity study. The MERA treated rats even in the highest concentration showed no significant change in the levels of renal toxicity markers like BUN, creatinine and total protein when compared with the control rats. The WBC count also showed no significant change (p > 0.05) between the control and MERA administered rats (Table 2).

3.3.3. Histopathological study

Histopathological analysis of the vital organs such as liver, heart and kidney of the MERA treated animals showed no altered histology and signs of toxicity. The liver showed normal architecture with a clear lumen of central vein and without any lesion or necrosis. Heart tissue of MERA treated rat showed no signs of toxicity. There was no renal injury evident in the histopathological evaluation of MERA treated rats. The renal tissues were free from any degeneration of Bowman’s space, glomeruli, proximal and distal tubules (Fig. 3).

| Table 1 | Effect of MERA (Dose in mg/kg body weight) on biochemical parameters in acute oral toxicity study. Values are expressed as mean ± standard deviation (n = 6 for each group). |
|---------|-------------------------------------------------------------------------------------------------|
| Relative organ weights | Biochemical parameters |
| Liver | Kidney | Heart | AST (U/L) | ALP (U/L) | ALT (U/L) |
| Control | 3.76 ± 0.03 | 0.93 ± 0.12 | 0.63 ± 0.03 | 135.01 ± 0.51 | 141.7 ± 0.82 | 44.27 ± 0.39 |
| MERA-550 | 3.65 ± 0.02 | 0.93 ± 0.06 | 0.61 ± 0.05 | 134.23 ± 0.84 | 141.1 ± 0.58 | 44.33 ± 0.85 |
| MERA-2000 | 3.71 ± 0.03 | 0.94 ± 0.03 | 0.60 ± 0.02 | 133.9 ± 0.86 | 140.8 ± 0.63 | 45.04 ± 0.52 |
4.3. Histopathological analysis of paw tissue

The histopathological analysis of paw tissue of both carrageenan (Fig. 4) and dextran (Fig. 5) treated group shows massive inflammation and edema formation compared to control rats. Inflammatory cell infiltration, proliferated epithelium, proliferated collagen, epidermal edema was markedly suppressed by MERA-100, MERA-200 and indomethacin administrated group as compared to dextran and carrageenan-treated group.

4. Discussion

Natural therapeutic drugs have become universally popular in primary aid, particularly in developing countries. According to the World Health Organization, 80% of the remote area population rely on traditional medicine and the history of medicinal plants used by the human as a medicine is about 60,000 years old [21]. Bioactive products from medicinal plants are likely to be safe without any compromising health effect, and thus widely used as self-medication [22]. However, the safety and toxicity assessment of herbal medicines is rarely done before their human consumption as they are considered to be inherently safe [23]. The clinical use of herbal drugs without adequate scientific evidence has raised concerns regarding their toxicity status [24]. Thus, toxicity evaluation of herbal medicines is being carried out in various experimental animal models to evaluate their safety for future human use [25,26]. Therefore the current study is designed to evaluate the safety assessment of MERA in an experimental rat model. The study also aims to evaluate the anti-inflammatory effect of MERA in in-vivo model.

*R. aquatica* belongs to the family Boraginaceae is represented by about 100 genera and 2000 species. The plant is scattered throughout Peninsular and Western Ghats of India in the sandy and rocky beds of streams and rivers often were occasionally submerged in floods. It is also distributed in Sri Lanka, China, tropical southeastern Asia, Africa, Brazil and Latin America. The plant is a necessary component of many Ayurvedic drug preparations since historical times. It is widely used as an crucial ancient drug for kidney and bladder stones.

Phenos and flavonoids are the major natural bioactive secondary metabolites in plants. These compounds are widely used all over the world to treat various diseases due to its biological properties like antioxidant, anti-carcinogen, anti-aging, protection from cardiovascular, immune/autoimmune diseases and brain dysfunctions viz. Parkinson's, Alzheimer's, Huntington's disease, etc [27,28]. The phytochemical evaluation of MERA shows the presence of secondary metabolites like alkaloids, flavonoids, phenolics and tannins, phytosterols, reducing sugars, proteins and terpenoids. The results of in-vitro antioxidant evaluation of MERA reveal its capability to scavenging free radical at a lower concentration.

In the acute toxicity study, a maximum dose of 2000 mg/kg b.wt. of the MERA caused neither signs of toxicity nor mortality during the 14 days of the experiment. Throughout the 14 day periods, all animals were found to be healthy with no changes in their skin and fur, eyes and mucous membranes, and behavioral patterns. Therefore, it is safe to propose that its oral LD50 value should be greater than 2000 mg/kg of body weight. The studies are performed according to OECD guidelines 423 [15].

Decreases or increases in the body weights are associated with toxic effects of chemicals and drugs. However, scientific evidence confirmed that increases or decreases in the body weights are accompanied by accumulation of fats and physiological adaptation responses to the plant extracts. The toxic effect of plant materials may leads to a decrease in appetite and lower the caloric intake by animal [29]. The relative weight of the vital organs like liver, kidney
and heart were found normal indicating no toxic effect in both control and treated group and was statistically non-significant differences (P > 0.05). All hematological parameters of MERA-extract supplemented rat were within the reference range and were comparable to that of control rat.

Some phytochemicals found in meditative plants is known to cause hepatotoxicity. This was evidenced by the presence of elevated levels of liver injury marker enzymes in the blood. In this light, plasma markers of hepatic damage such as AST, ALT and ALP were assayed to investigate the possible hepatototoxicity of MERA extract. There was no significant alteration could be recorded for AST, ALT, or ALP, suggesting no inflammatory or necrotic damage of liver due to MERA extract. Histopathological observation of sections of the liver of MERA-treated rat attests to the fact that, there is no hepatic damage as marked by the normal looking integrity of hepatocytes.

The kidney is a sensitive organ whose function is known to be suffered from a number of factors such as drugs including phytochemicals of plant origin that ultimately lead to renal failure [30]. Assessment of possible renal damage due to MERA extract was made by assaying blood urea nitrogen (BUN), total protein and creatinine levels. Results show no significant alteration in the creatinine and BUN level due to MERA treatment. Histological observation has showed no major toxic manifestation in the cortical or medullary areas of the kidney. The histological architecture of glomeruli and tubules in control and MERA extract treated rat being comparable, the nontoxic nature of MERA extract stands validated.

Several experimental protocols of inflammation and pain are used for evaluating the potency of drugs. In the present study, the evaluation of anti-inflammatory effect was undertaken using an animal model to fully investigate the potential of MERA to be used in the treatment of inflammatory disorders.

The carrageenan-induced inflammatory processes are biphasic [31]. The initial phase (first hour) is attributed to the release of histamine and serotonin [32] and the second accelerating phase of swelling is due to the release of prostaglandin, bradykinin and lysozyme. The second phase of edema formation was sensitive to both steroidal and non-steroidal agents [33]. In the current study, edema inhibition by MERA was more prominent at the second phase and it could be due to the inhibition of prostaglandin production by MERA via exerts an anti-inflammatory effect. Another mechanism of action by MERA could be due to suppression of the release of histamine, serotonin and bradykinin which are prominent mediators involved in the primary and secondary phase of carrageenan-induced edema formation [34]. Histopathological analysis of paw tissue of rat shows the inhibition of inflammation and edema formation in

![Fig. 3. Histopathology of liver (A,B,C), heart (D,E,F) and kidney (G,H,I) of control and MERA treated rats in the acute toxicity study for 14 days. A, D, G: Control rat; B, E, H: MERA-550 mg/kg b.wt; C, F, I: MERA-2000 mg/kg b.wt.](image-url)
MERA treated group as compared to the carrageenan-treated group. The paw tissue of the normal rats showed no signs of inflammation (Fig. 4a) with normal keratin, Sub epidermal layer, Sub cutaneous layer. In the rats treated with carrageenan shows massive influx of inflammatory cell infiltration, Proliferated collagen, Hyper keratotic skin, Sub epidermal edema (SEE). Treatment with MERA showed marked improvement in the inflammatory signs. Keratinization was decreased and dermis and hypodermis appeared to be normal. Mild sub epidermal edema, mild epithelial hyperplasia and mild inflammation were also observed in MERA treated group (Fig. 4d–e).

Dextran is a polysaccharide of high molecular weight that induces anaphylactic reaction. It was characterized by extravasation and edema formation, as consequence of liberation of histamine and serotonin from mast cells [35]. There was a significant reduction (p < 0.05) in dextran-induced rat paw edema in all groups, at 1st, 2nd and 3rd hours of the experiment at a concentration of 200 mg/kg.b.wt. Histopathology of paw tissue also reveals the reduction of inflammation and edema formation in MERA treated group as compared to the dextran treated group.

The histopathological findings also substantiate with the paw edema analyses. Histopathological studies indicated that inflammatory cell infiltration, edema formation and hyperkeratinisation were markedly suppressed in the rats treated with the MERA. This evidence shows the efficacy of MERA as an anti-inflammatory agent.

Fig. 4. Histology of rat paw tissue (H&E stain 40×) in carrageenan-induced paw edema. Values expressed as average of 3 samples ± SEM in each group. a: Control; b: Carrageenan; c: Indomethacin; d: MERA-100 mg/kg b.wt; e: MERA-200 mg/kg b.wt. a) Cross section of normal paw tissue shows keratin (KR), Sub epidermal layer (SEL), Sub cutaneous layer (SC). b) Cross section of carrageenan induced rat paw tissue shows Massive influx of inflammatory cell infiltration (ICI), Hyper keratotic skin (HKS), Sub epidermal edema (SEE). c) Cross section of tissue of carrageenan + diclofenac sodium shows sub epidermal edema (SEE), Mild epithelial hyper plasia (EHP). d) Cross section of the paw tissue of carrageenan + MERA (100 mg/kg b.wt) shows Mild edema (ME) and Mild inflammation (MI). e) Cross section of paw tissue of carrageenan + MERA (200 mg/kg b.wt) shows keratin (KR), Mild epithelial hyper plasia (EHP), Mild Sub epidermal edema (MSEE).
5. Conclusion

The present study confirms the anti-inflammatory effect of MERA and its safety usage up to the dose of 2000 mg/kg of body weight in an experimental rat. The further studies are needed to reveal the exact mechanism of action of the MERA. A systemic research is needed to produce a nutraceuticals drug from roots of *R. aquatica* Lour. for treating various health problem of humankind.

Sources of funding

None.

Conflict of interest

None.

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