



Acid Uric 3× Tablet: Standardization and pharmacological evidence of uric acid use as anti-gout medicine

[Acid Uric 3× Tablet: estandarización y evidencia farmacológica del uso de ácido úrico como medicamento contra la gota]

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Abstract

Context: Despite being a causative agent of gout, uric acid is used as an anti-gout medicine in homeopathy without reported scientific evidence, and such highly diluted medicines lack the exact amount of active ingredient per dose, which is mandatory for pharmacological evaluation.

Aims: To determine active contents per tablet and evaluate anti-gout and anti-inflammatory activities of Acid Uric 3× Tablet, a homeopathic anti-gout medicine.

Methods: A simple RP-HPLC method was developed and validated for the determination of uric acid in Acid Uric 3× Tablet. The standardized tablet was then investigated for anti-gout activity using xanthine oxidase inhibition and potassium oxonate-induced hyperuricemia in rats models. The tablet was also investigated for anti-inflammatory activity employing both *in vitro* and *in vivo* models.

Results: The developed HPLC method was found to be simple, sensitive and precise. Acid Uric 3× Tablet was found to contain 250 µg uric acid/tablet. This medicine inhibited xanthine oxidase activity ($IC_{50} = 12.42 \mu\text{g/mL}$) and lowered the serum uric acid in tablet-treated rats (1.02 mg/dL) as compared to toxic group (4.87 mg/dL). The tablet also showed anti-inflammatory activity in heat-induced protein denaturation ($IC_{50} = 4.6 \pm 1.0 \mu\text{g/mL}$), anti-proteinase ($IC_{50} = 4.64 \pm 1.0 \mu\text{g/mL}$), heat-induced RBC hemolysis ($IC_{50} = 12.39 \pm 1.5 \mu\text{g/mL}$) and hypotonicity-induced hemolysis ($IC_{50} = 11.31 \pm 1.0 \mu\text{g/mL}$). Moreover, significant anti-inflammatory activity was found in the carrageenan-induced rat-paw edema model at a dose of 0.132 mg/kg.

Conclusions: The findings of the study indicate that the developed HPLC method may be used to standardize Acid Uric 3× Tablet and provide scientific evidence of lowering uric acid level.

Keywords: alternative medicine; biological fluids; homeopathy; RP-HPLC; uric acid.

Resumen

Contexto: A pesar de ser un agente causal de la gota, el ácido úrico se usa como un medicamento contra la gota en la homeopatía sin evidencia científica reportada, y tales medicamentos altamente diluidos carecen de la cantidad exacta de ingrediente activo por dosis, lo cual es obligatorio para la evaluación farmacológica.

Objetivos: Determinar los contenidos activos por tableta y evaluar las actividades anti-gota y anti-inflamatorias de Acid Uric 3× Tablet, un medicamento homeopático contra la gota.

Métodos: Se desarrolló y validó un método RP-HPLC simple para la determinación de ácido úrico en Acid Uric 3× Tablet. Luego se investigó la tableta estandarizada para determinar la actividad antigota usando la inhibición de la xantina oxidasa y la hiperuricemia inducida por oxonato de potasio en modelos de ratas. La tableta también se investigó para determinar la actividad anti-inflamatoria empleando modelos *in vitro* e *in vivo*.

Resultados: El método HPLC desarrollado fue simple, sensible y preciso. Acid Uric 3× Tablet contenía 250 µg de ácido úrico/tableta. Esta inhibió la actividad de la xantina oxidasa ($IC_{50} = 12,42 \mu\text{g/mL}$) y redujo el ácido úrico en suero de ratas tratadas con tabletas (1,02 mg/dL) en comparación con el grupo tóxico (4,87 mg/dL). La tableta también mostró actividad anti-inflamatoria en la desnaturalización de proteínas inducida por calor ($IC_{50} = 4,6 \pm 1,0 \mu\text{g/mL}$), antiproteínasa ($IC_{50} = 4,64 \pm 1,0 \mu\text{g/mL}$), hemólisis de glóbulos rojos inducida por calor ($IC_{50} = 12,39 \pm 1,5 \mu\text{g/mL}$) y hemólisis inducida por hipotonicidad ($IC_{50} = 11,31 \pm 1,0 \mu\text{g/mL}$). Además, se encontró una actividad anti-inflamatoria significativa en el modelo de edema plantar en rata inducido por carragenano a una dosis de 0,132 mg/kg.

Conclusiones: Los resultados del estudio indican que el método de HPLC desarrollado puede usarse para estandarizar la tableta de ácido úrico y proporcionar evidencia científica de la reducción del nivel de ácido úrico.

Palabras Clave: medicina alternativa; fluidos biológicos; homeopatía; inflamación; RP-HPLC; ácido úrico.

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INTRODUCTION

Xanthine, a purine, is converted into uric acid by the activity of xanthine oxidase. The normal concentration of uric acid in the body is 3.4 - 7.0 mg/dL in males and 2.4 - 6.0 mg/dL in females, and it acts as an anti-inflammatory and xanthine oxidase inhibitory at a normal physiological concentration as reported by numerous scientific studies (Lai et al., 2017). Uric acid is deposited as monosodium urate in joints and kidneys due to either impaired xanthine metabolism or consumption of a purine-rich diet. Hence, uric acid is an important diagnostic marker of gouty arthritis, type-II diabetes, hyperlipidemia, cardiovascular disorders and kidney diseases (Lin et al., 2000). Moreover, xanthine oxidase generates superoxide free radicals, which involve various types of inflammatory diseases and vascular/tissue injuries (Berry and Hare, 2004; Rohman et al., 2010). Thus, the risk of gout can be reduced by decreasing biosynthesis or increasing excretion of uric acid from the body (Umamaheswari et al., 2007). Xanthine oxidase inhibitors such as allopurinol block uric acid biosynthesis hence used clinically to treat gout (Pacher et al., 2006). Allopurinol is quite effective, but it generates superoxide radicals and causes allergic reactions, rash, liver function abnormalities and hypersensitivity syndrome that warrants to opt alternative treatments, including homeopathic medicines (Umpierrez et al., 1998). Numerous attempts have been made to find safer alternative therapy for curing gout (Kong et al., 2004; Zhu et al., 2004).

Uric acid in highly diluted form is used as a therapeutic agent in the Homeopathic System of Medicine (O'Connor, 1883; Bayani, 2016; Lenger, 2018). This use is based on the principle of therapeutic similitude, a substance that causes disease symptoms in large doses can cure similar symptoms in minute doses (Teixeira, 2011). Uric acid, named as *Uricum Acidum* in homeopathy, is commonly used in the homeopathic system for the treatment of gout (Clark, 1993; Boericke, 2001). However, repeated intake of uric acid may aggravate the disease, which needs to be investigated

scientifically. Therefore, the present study aimed to evaluate Acid Uric 3× Tablets for anti-gout and anti-inflammatory activities.

For pharmacological evaluation, the compound or dosage form must be having an accurate or fixed amount of active ingredients. High dilution and complex nature of homeopathic medicine are posing challenges in the quantification of active ingredients (Daharwal and Shrivastava, 2019). Hence, there is a need to develop sensitive methods using modern analytical tools. Acid Uric 3× Tablet contains uric acid as an active ingredient, the amount of which per tablet is not mentioned, which is mandatory prior to conduct a pharmacological evaluation.

The literature review indicated several methods for the determination of uric acid including enzyme-catalyzed uric acid oxidation and subsequent detection of the resulting chromophore, colorimetric detection utilizing various reagents, electrochemical detection utilizing electrodes and biosensors and reversed-phase HPLC methods (Jelick-Stankov et al., 2003; Dai et al., 2007; Piermarini et al., 2013; Tanaka et al., 2013). However, such methods are cumbersome, laborious and need the carcinogenic nature of reagents (Jelick-Stankov et al., 2003; Dai et al., 2007). Therefore, there is a need for a simple analytical method for the determination of uric acid in Acid Uric 3× Tablet. Keeping these points in view, the present study aimed to develop a simple method to quantify Acid Uric 3× Tablet before performing anti-inflammatory and anti-gout activities. The findings of the present study may provide a method for the standardization of Acid Uric 3× Tablet and scientific evidence to its anti-gout usage.

MATERIAL AND METHODS

Materials

Uric acid (Difco Laboratories, USA), Acid Uric 3× Tablet (Batch No 25, BM Homeo Pakistan), xanthine oxidase (Sigma-Aldrich), xanthine (Bioworld, Biofine Plus Research Chemicals), allopurinol (Toshiba, Tokyo, Japan), potassium oxonate (Alfa

Aesar), HPLC grade methanol and acetonitrile, trypsin, casein, perchloric acid, hydrochloric acid, hydrocortisone sodium, diclofenac sodium, TRIS HCl buffer, acetic acid, sodium acetate, sodium chloride and sodium hydroxide (Merck, Germany) were procured from the local market. Other materials included RBC's taken from healthy human volunteers who had not taken any non-steroidal anti-inflammatory drugs (NSAIDs) and in-house prepared double distilled water.

Instruments

Beckman Coulter AU480 (USA), homogenizer (Wisestir Homogenizer Stirrer-HS-30E, Korea), refrigerator centrifuge machine, Sigma 2-16 Kc, Germany, digital plethysmometer (LE 7500, Panlab, Harvard apparatus) and liquid chromatographic system (1200 series, Agilent Technologies, Waldronn, Germany) having isocratic pump (G1310 A), auto-sampler (G1329 A), column thermostat (G1316 A) and diode array detector (G1315 B) were used in the present study.

Determination of uric acid by RP-HPLC

Standard solutions

Uric acid standard stock solution (500 µg/mL) was prepared in 1% aqueous sodium acetate. Then, a range of working standard solutions (0.25-100 µg/mL) was prepared by diluting the stock solution with distilled water.

Mobile phase

The mobile phase was prepared mixing 324.1 mL of 0.2 M acetic acid and 25.9 mL of 0.2 M sodium acetate and making the volume 350 mL with water. The pH was adjusted to 3.6 with 1 N hydrochloric acid, and the volume was made with 700 mL double distilled water.

Chromatographic conditions

The standard solution (20 µL) was eluted through a column (Agilent 5 TC-C₁₈ (2) 250×4.6 mm) at a flow rate of 0.8 mL/min using the mobile phase. The temperature of the column was maintained at 35°C, and detection was carried out at

300 nm using DAD. The peak obtained was used to determine the system suitability (Wiggins, 1991).

System suitability

The system suitability was ensured by determining tailing factor, capacity factor (*k'*), peak asymmetry, number of theoretical plates (*N*), and height equivalent to a theoretical plate (HETP) (Wiggins, 1991).

Method validation

Briefly, uric acid standard solutions having concentrations (0.25 - 100.00 µg/mL) were analyzed in triplicate to construct a plot of concentration versus peak area, and linearity was evaluated by visual inspection of the plot and applying the linear regression and correlation coefficient (Karnes and March, 1991). Recovery was evaluated spiking Acid Uric 3× Tablet with three standard solutions (2.5, 5.0 and 10.0 µg/mL). Intra-day and inter-day accuracy and precision were determined using three standard solutions (2.5, 5.0 and 10.0 µg/mL), which were analyzed 6 times in a single day and once daily for six consecutive days, respectively. The relative standard deviation of the six data points was taken as precision. The sensitivity, LOD and LOQ were determined statistically using five standard solutions of concentrations 2.50 - 40.0 µg/mL. The calibration curves were constructed, and the mean slope (*S*) and the standard deviation of the intercepts (*σ*) were used to calculate LOD and LOQ. Robustness of the method was determined by changing pH (3.6 ± 0.1), detection wavelength (300 ± 2 nm) and column temperature (35 ± 2°C).

Determination of active content in Acid Uric 3× Tablet

Twenty Acid Uric 3× Tablets were taken and ground to a fine powder. Tablet powder equivalent to the weight of one tablet (250 mg) was dissolved in 20 mL of 1% sodium acetate solution and analyzed by HPLC. Peak area was used to determine uric acid contents from the linear regression equation, obtained from the calibration curve (Zuo et al., 2008).

Pharmacological studies

In vitro anti-gout activity (xanthine/xanthine oxidase inhibition)

Xanthine/xanthine oxidase inhibition assay was used to determine anti-gout activity (Sunarni et al., 2015). Briefly, a test sample solution was prepared mixing phosphate buffer (50 mM; 300 μ L) having pH 7.5. Then, 100 μ L of each standard/sample solution, xanthine oxidase enzyme solution (0.2 units/mL) and distilled water were mixed. A control was prepared using 100 μ L of phosphate buffer instead of the test sample. The test solution and control were incubated for 15 min at 37°C, and then 1 mM xanthine solution (2 mL) was added, and the contents were again incubated for 30 min. One milliliter of 1 N HCl was added in the reaction mixture to arrest the reaction followed by determination of absorbance at 287 nm using phosphate buffer as blank. The enzyme inhibition activity was determined using the following equation [1]:

$$\text{Xanthine oxidase inhibition (\%)} = \left(1 - \frac{\beta}{\alpha}\right) \times 100 \quad [1]$$

Where, α represents the enzyme activity without test drug and β represents the activity of the enzyme with the test drug.

In vitro anti-inflammatory activity

Inhibition of egg albumin denaturation

Egg albumin denaturation inhibition activity was estimated following the method reported in the literature with some modifications (Chandra et al., 2012). A test solution comprising 4% filtered egg albumin solution (4 mL) and standard/sample solution (1 mL) was incubated (37°C) for 20 min and then transferred to a water bath (75°C) for 5 min. A control solution was prepared like the samples with the exception that the compound's solution was replaced with distilled water and treated like the sample. The contents were cooled, and absorbance was determined at wavelength 660 nm using distilled water as blank.

Antiproteinase activity

The activity was determined using a method described earlier with some modifications (Leelaprakash and Dass, 2011). The reaction mixture comprising 1 mL of standard/sample solution, trypsin (0.06 mg), 1 mL of Tris hydrochloride buffer (20 mM; pH 7.4) was incubated (37°C) for 5 min. Then, 0.8% casein solution (1 mL) was added, followed by incubation (37°C) for 20 min. Two milliliters perchloric acid (70%) was added in the reaction mixture to arrest the reaction. A control solution was prepared like the samples with the exception that compound's solution was replaced with distilled water and treated like the sample. The reaction mixture was centrifuged (2500 rpm) for 15 min to obtain supernatant whose absorbance was determined at wavelength 210 nm using distilled water as blank.

Membrane stabilization against heat-induced hemolysis

This activity was determined following the method reported in the literature (Sakat et al., 2010). The experimental protocol utilized in the current study were authorized and approved by the Human Ethical Committee, Vide reference No. HEC/PUCP/1978, Punjab University, Lahore, Pakistan. Blood from the healthy human volunteer (which has not taken NSAIDs for two weeks before the experiment) was centrifuged (3000 rpm) for 10 min. RBC's separated from the blood were washed thrice using an equal volume of normal saline followed by reconstitution with buffer (pH 7.4) to get 10% v/v RBC's suspension. A test solution containing 2 mL standard/sample and 2 mL 10% RBC suspension was placed in a thermostatically controlled water bath maintained at 56°C for 30 min. A control prepared replacing sample solution by 0.9% normal saline was treated like the sample. Afterward, the contents were cooled, centrifuged (2500 rpm) for 10 min, followed by measurement of absorbance of supernatants at wavelength 560 nm using distilled water as blank.

Membrane stabilizing against hypotonicity induced hemolysis

This activity was determined following the method reported in the literature (Azeem et al., 2010). A reaction mixture containing standard/sample solution (1 mL), hyposaline solution (2 mL; 0.25% w/v NaCl), phosphate buffer having pH 7.4 (1 mL) and RBC suspension (0.5 mL) was incubated (37°C) for 30 min and centrifuged (3000 rpm) for 20 min to obtain supernatant whose absorbance was determined at wavelength 560 nm using distilled water as blank. A control was prepared replacing sample solution by hyposaline and treated like the sample. The absorbances of the control and sample were used to determine percent activity in egg albumin denaturation inhibition assay, proteinase inhibition assay, membrane stabilization assay against heat and hypotonicity induced hemolysis using the following equation [2]:

$$\text{Activity (\%)} = \text{Absorbance of control} - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad [2]$$

***In vivo* studies**

Laboratory animals

Forty-eight male Wistar rats, weight 190 ± 15 g, aged 15 weeks, acquired from the Animal House, University College of Pharmacy, Lahore, Pakistan, were housed to acclimatize to 12 hours light/dark cycle at room temperature (25°C) with constant humidity for one week period. Rodent pellet-diet (15 - 25 g/day) was provided and water (30 - 50 mL) was supplied *ad libitum* to experimental animals. The experimental procedures utilized in the current study were authorized and approved by the Bioethics Committee, Vide reference No. D/1304/02, Punjab University, Lahore, Pakistan.

Preparation of dose

A dose of 0.132 mg/kg was selected for *in vivo* anti-gout and anti-inflammatory studies of Acid Uric 3× Tablet, and it was determined on the basis of the following formula i.e. Animal equivalent dose (mg/kg) = (human equivalent in mg/kg) (K_m of human/ K_m of rat) (Nair and Jacob, 2016). Doses of Acid Uric 3× Tablet, potassium oxonate, allopurinol and diclofenac sodium were prepared in a mixture of water and Tween 80 (95:5 v/v), which were administered perorally.

rinol and diclofenac sodium were prepared in a mixture of water and Tween 80 (95:5 v/v), which were administered perorally.

Experimental design and treatments

The *in vivo* anti-gout activity was determined following the method described in the literature with some modifications (Nguyen et al., 2017). The anti-gout activity protocol was designed into two parts: protective and curative. For the former, thirty male Wistar rats were segregated into four groups, six rats per group, except group-II, which had twelve rats. These followed the subsequent order: Group I (uncompromised and untreated, vehicle control, 10 mL/kg, perorally), group II (toxic, potassium oxonate, 250 mg/kg, perorally), group III (Acid Uric 3× Tablet, 0.132 mg/kg, perorally) and group IV (allopurinol, 10 mg/kg, perorally). To induce hyperuricemia, uricase inhibitor (potassium oxonate) was administered by oral gavage (p.o.) one hour prior to administration of test samples in all treatment groups (II, III and IV) except control (group-I) and the study was continued for 21 days for protective study design.

Irrespectively, whereas for the later (curative study), nine rats separated from group-II after protective study period were randomly segregated into three treatment groups (n=3) receiving group-wise treatments as follows: Group-I (vehicle control, 10 mL/kg, perorally), group-II (Acid Uric 3× Tablet, 0.132 mg/kg, per orally) and group-III (allopurinol, 10 mg/kg, perorally), respectively. The doses were administered for 7 days in the curative study.

The *in vivo* anti-inflammatory activity was determined following the method reported in the literature (Muhammad et al., 2012). For *in vivo* anti-inflammatory activity, eighteen rats were segregated into three groups (each n = 6) with group-wise treatments given as follows: Group-I (vehicle control, 10 mL/kg, perorally), group-II (Acid Uric 3× Tablet, 0.132 mg/kg, perorally) and group-III (diclofenac sodium, 10 mg/kg, perorally), respectively. There is edema induction in hind paw of Wistar rats by subplantar injection of 1.0 % carrageenan (0.1 mL) immediately 1 h after the admin-

istration of test drug. Swelling in hind-paw was measured using a digital plethysmometer (LE 7500, Panlab, Harvard Apparatus, Barcelona, Spain) immediately before drug administration and later at 1, 2, 3, 4 and 24 h. Percent edema inhibition was calculated in comparison with the control group using the following formula [3]:

$$\text{Percent inhibition} = A - \frac{B}{A} \times 100 \quad [3]$$

Where A represents the edema volume of the control group and B as edema volume of the treated group.

Collection of blood and tissues

One hour after the last dose administration of the treatment in protective and curative study, the blood samples were collected in plain tubes by cardiac puncture of experimental rats under diethyl ether anesthesia. Then animals were euthanized under diethyl ether anesthesia to harvest liver tissue, which was washed with 0.9% normal saline. The liver homogenate was prepared by mincing liver tissue (150 mg) in a sterile Petri dish, kept on an ice pack. Then the minced tissue was suspended in an ice-cold 50 mM phosphate buffer (3 mL; pH 7.4) and then homogenized for 5 min. The tissue homogenate was centrifuged (refrigerator centrifuge machine, Sigma 2-16 Kc, Germany) at 3000 g for 10 min at 4°C to collect the supernatant. The kidney of each experimental animal was preserved in neutral buffered formalin (10% v/v).

Biochemical analysis and histology

To collect serum, blood samples were centrifuged (2700 rpm) for 10 min, and serum samples were analyzed for uric acid by Beckman Coulter (AU 480 Chemistry Analyzer, Beckman Coulter, Inc, CA, USA). For histopathology, kidney samples were treated by routine techniques of dehydration in alcohol and paraffin embedding. Sections (4-5 μm thickness) were made and stained with hematoxylin and eosin stains followed by histopathological examination of slides under a light microscope (Olympus microscope, Japan). The light microscope was equipped with a digital microscope camera (DMC) at 100× magnification.

Xanthine oxidase activity of liver homogenate

A reaction mixture containing 0.5 mL supernatant, 3.5 mL phosphate buffer solution (pH 7.5) and 1 mM potassium oxonate (1 mL), was incubated at 37°C for 15 min and then 0.250 mM xanthine (1 mL) was added and contents were further incubated (37°C) for 30 min. The reaction was arrested by 0.5 M HCl (0.5 mL), followed by the determination of absorbance at 290 nm. The xanthine oxidase activity was expressed as nM of uric acid produced/min/mg of protein (Mo et al., 2007).

Statistical analysis

The results were mentioned as mean ± standard deviation after analysis of all the standards/samples in triplicate. The data were analyzed statistically by ANOVA (one-way) with PostHoc multiple comparisons with Bonferroni using SPSS 22.0 (IBM SPSS Statistics). A p<0.05 was regarded as significantly different. IC₅₀ (half maximal inhibitory concentration) was determined applying linear regression on dose-response curves. These were named as half maximal inhibitory concentration (IC₅₀; μg/mL) of the drug to inhibit enzyme activity using xanthine oxidase and proteinase activities, to inhibit albumin denaturation, RBC's hemolysis using heat and hypotonicity induced hemolysis inhibition assay, respectively.

RESULTS

Development of RP-HPLC method

Different mobile phases comprising pure methanol, water, mixtures of water and methanol in different proportions (5:95, 50:50, v/v) and aqueous buffered solutions of various compositions and pH (monobasic potassium phosphate buffer pH 3.0, sodium phosphate buffer pH 4 and sodium phosphate buffer pH 7) were used to elute the sample through C₁₈ column. However, the mobile phase comprising aqueous acetate buffer (pH 3.6) gave the symmetrical/Gaussian peak (Fig. 1A). Hence, the peak obtained was used to assess the system suitability.

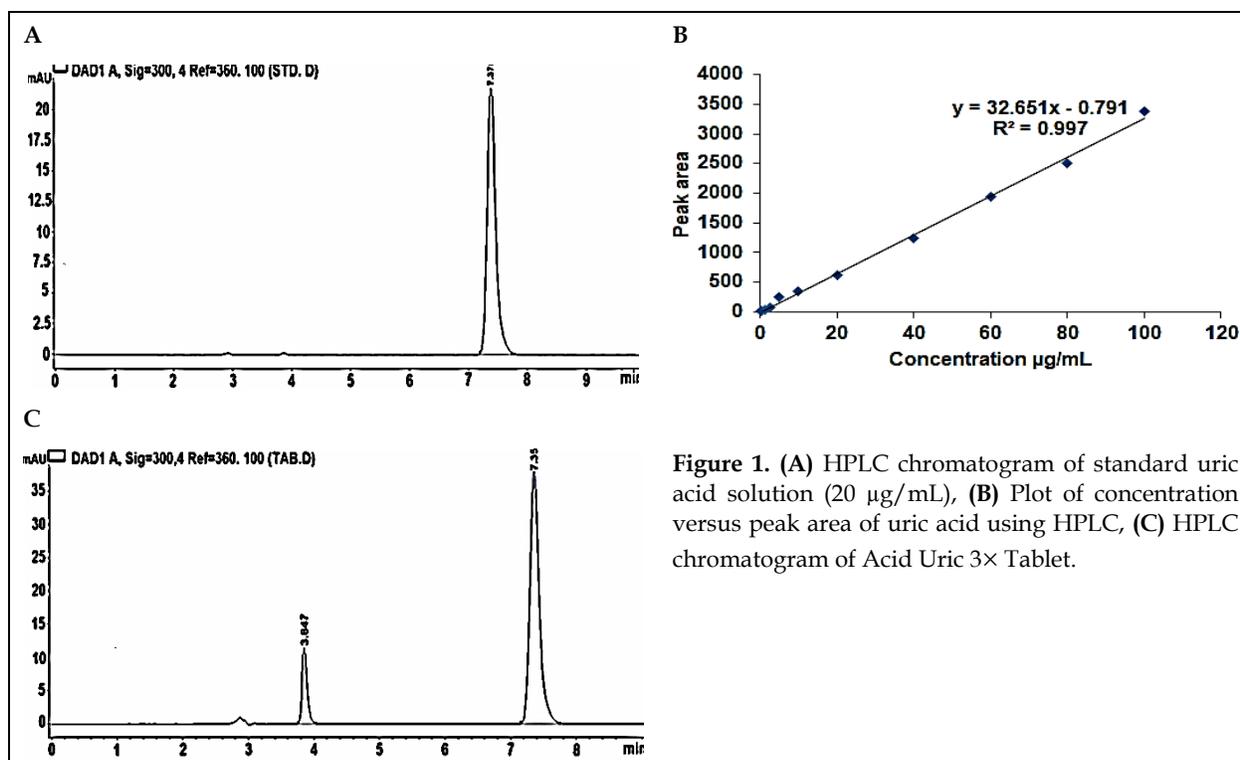


Figure 1. (A) HPLC chromatogram of standard uric acid solution (20 µg/mL), (B) Plot of concentration versus peak area of uric acid using HPLC, (C) HPLC chromatogram of Acid Uric 3× Tablet.

Table 1. System suitability parameters calculated from chromatogram of uric acid.

Parameters	Value	Acceptable limit
Capacity (retention) factor	3.61	$K \geq 2$
Peak asymmetry/Tailing factor	1.0	$T \leq 2$
Number of theoretical plates	21762.15	$N > 2000$
Height equivalent to theoretical plate	11.48 µm	The smaller value, the higher column efficiency

Table 2. Recovery, intra- and inter-day accuracy and precision of RP-HPLC method for determination of uric acid.

Concentration (µg/mL)	Recovery (n = 3) (%) ± SD	Intraday (n = 6)		Interday (n = 6)	
		Accuracy (%) ± SD	Precision (RSD%)	Accuracy (%) ± SD	Precision (RSD%)
2.5	105.27 ± 0.42	105.54 ± 0.23	0.27	105.49 ± 0.48	0.56
5	98.67 ± 0.60	106.45 ± 1.58	0.91	108.6 ± 0.94	0.53
10	101.43 ± 0.65	101.98 ± 1.92	0.57	101.81 ± 1.7	0.54

SD: standard deviation; RSD: relative standard deviation; values were expressed as mean ± SD.

System suitability

The system suitability parameters calculated are given in Table 1. These parameters were found to be within the specified limits, which indicated that the chromatographic system was suitable for the determination of uric acid, hence the method was subjected to validation.

Method validation

The plot of concentration *versus* peak area (Fig. 1B) indicated that the method was linear over the whole range investigated (0.25 - 100.0 µg/mL). The linear regression equation, the sum of squares (37,895,428.158), the sum of squares residual (74,976.932) mean square (37,895,428.158), mean square residual (10,710.990) and correlation coefficient ($R^2 = 0.997$) confirmed the linearity of the method ($p = 0.000$). The linear studies indicated the useful range to be 0.25 - 100.0 µg/mL, wherein the Beer's Law was obeyed. The recovery, intra-day and inter-day accuracy and precision, determined at three different concentration levels (2.5, 5.0, 10.0 µg/mL), are given in Table 2. The recovery (98.6 - 105.2%, SD < 5%), intraday accuracy (101.98 - 106.45%, RSD < 5%) and inter-day accuracy (101.81 - 108.6%, RSD < 5%), indicated that the method was reliable, repeatable and reproducible. The LOD and LOQ were found to be 0.187 and 0.62 µg/mL, respectively. Furthermore, the method was found to be robust as a slight variation in mobile phase pH (± 0.1), detection wavelength (± 2 nm), and column temperature ($\pm 2^\circ\text{C}$) did not affect the accuracy.

Uric acid in Acid Uric 3× Tablet

The chromatogram of Acid Uric 3× Tablet shown in Fig. 1C was used to determine uric acid contents. The peak of the sample was identified comparing the retention times of the sample peak and the standard peak. This chromatogram indicated that the peak of uric acid was not affected by any other peak due to the tablet matrix. The tablet was found to have 0.250 mg of uric acid per unit.

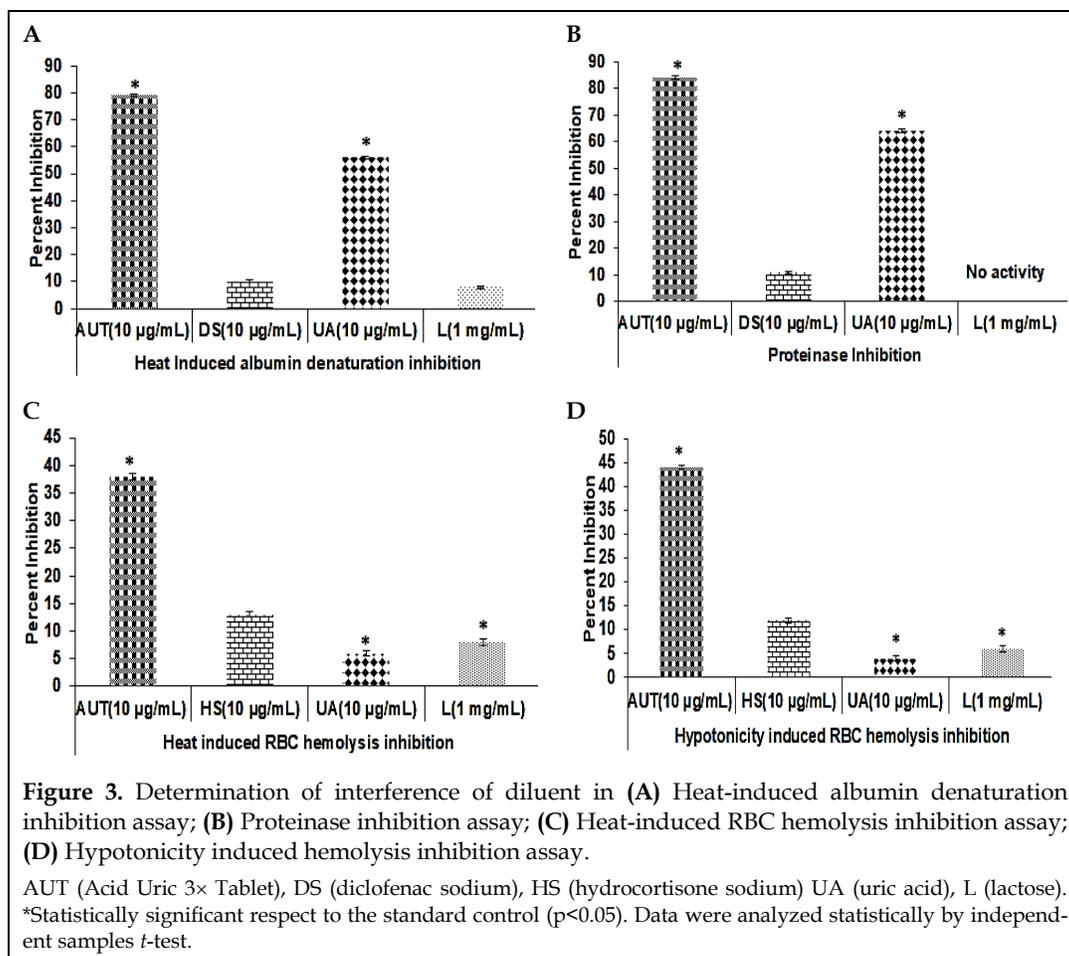
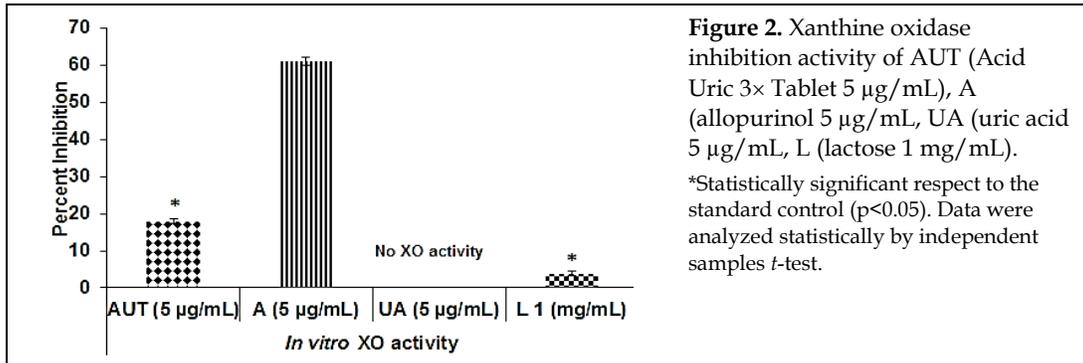
Pharmacological studies

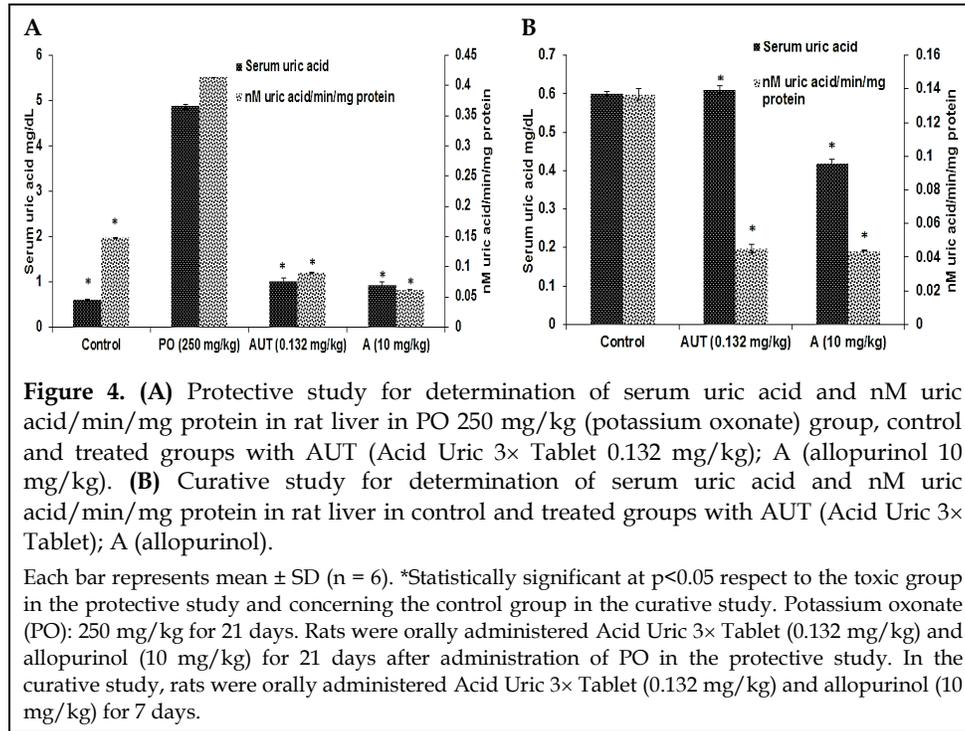
In vitro studies

The results of xanthine oxidase inhibition (XOI) activity of Acid Uric 3× Tablet and allopurinol at an equivalent concentration (5.0 µg/mL) are given in Fig. 2. These results indicated that the activity of the tablet and allopurinol were significantly different from each other ($p < 0.05$). In homeopathic, 3× attenuation tablet contains one part of an active ingredient, which is diluted thrice with ten parts of an inert diluent, lactose (Taub et al., 2017). Hence, to rule out the involvement of lactose, the diluent of the tablet and uric acid, lactose solution (1 mg/mL) and pure uric acid (5 µg/mL) were also investigated for XOI activity. The half maximal inhibitory concentration (IC_{50}) of Acid Uric 3× Tablet determined in a concentration range 2.5 - 12.5 µg/mL was found to be 12.42 µg/mL ($y = 4.0941x - 0.8824$, $R^2 = 0.9942$).

The results of the anti-inflammatory activity of Acid Uric 3× Tablet and standard at an equivalent concentration (10.0 µg/mL) are given in Fig. 3. To rule out the involvement of lactose, the diluent of the tablet, in the anti-inflammatory activity of the Acid Uric 3× Tablet, lactose solution having concentration (1 mg/mL) and pure uric acid (10 µg/mL) were also investigated for the anti-inflammatory activity along with standard drug (10 µg/mL) and Acid Uric 3× Tablet (10 µg/mL). In all three models, at a concentration of 10 µg/mL, the Acid Uric 3× Tablet inhibited 79.00% of heat-induced egg albumin denaturation as compared with 10.00% produced by diclofenac sodium, 84.00% of proteinase inhibition as compared with 11.00% produced by diclofenac sodium, 38.00% of heat-induced RBC hemolysis inhibition as compared with 13.00% produced by hydrocortisone sodium and 44.00% of hypotonicity induced hemolysis as compared with 12.00% produced by hydrocortisone sodium (Fig. 3).

The dose-dependent activity studies of Acid Uric 3× Tablet indicated IC_{50} at 4.6 µg/mL in heat-





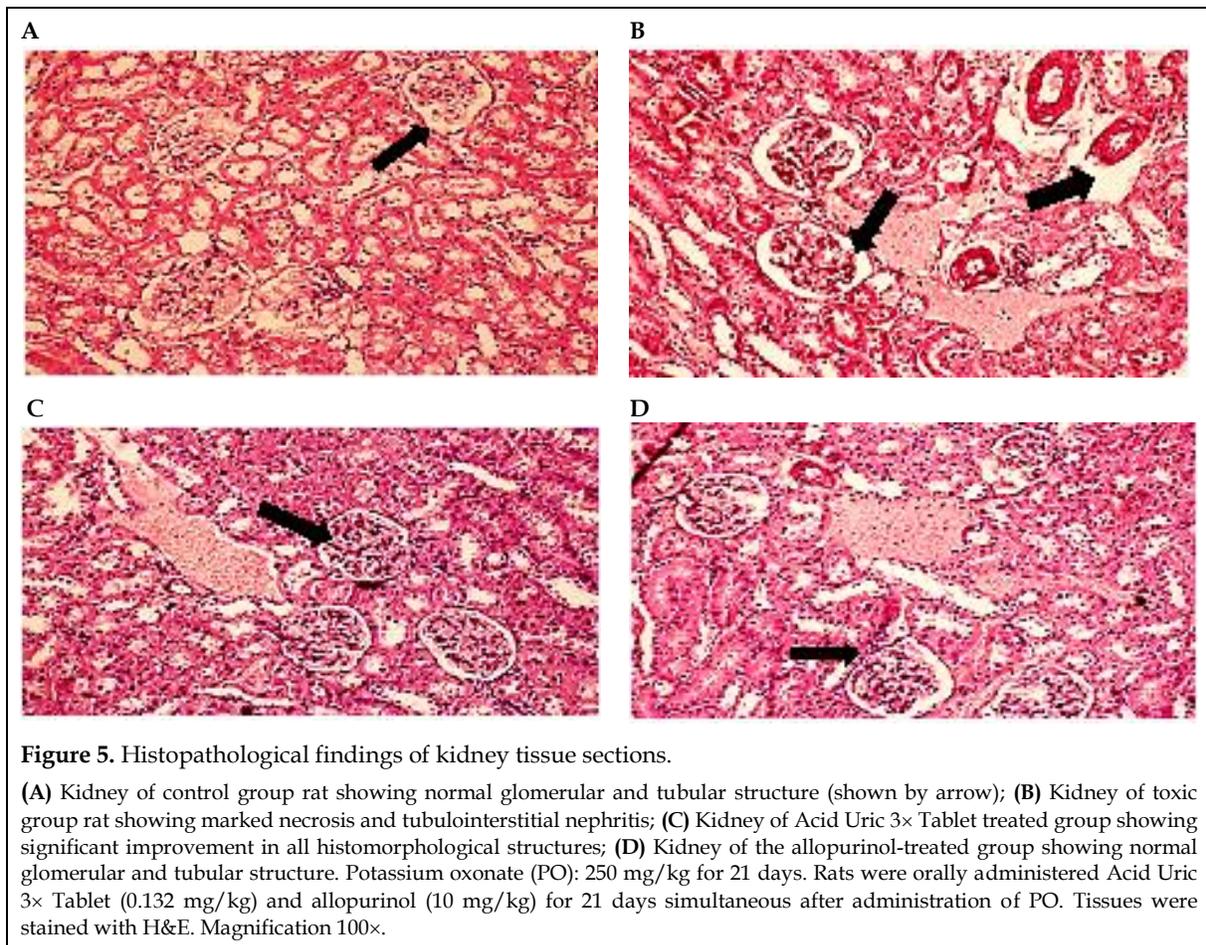
induced albumin denaturation (linear regression equation, $y = 5.6694x + 23.836$, $R^2 = 0.9933$), 4.64 $\mu\text{g}/\text{mL}$ in anti-proteinase activity ($y = 6.236x + 21.036$, $R^2 = 0.9912$), 12.39 $\mu\text{g}/\text{mL}$ in heat-induced RBC hemolysis inhibition assay ($y = 3.9461x + 1.0794$, $R^2 = 0.9918$) and 11.31 $\mu\text{g}/\text{mL}$ in hypotonicity-induced RBC hemolysis inhibition assay ($y = 4.1145x + 3.4478$, $R^2 = 0.9987$).

In vivo studies

The results of the protective anti-gout activity of Acid Uric 3× Tablet assessed by serum uric acid level and xanthine oxidase activity of liver homogenate are given in Fig. 4A. Administration of uricase inhibitor (potassium oxonate) resulted in significant hyperuricemia, as indicated by an increase in the serum uric acid level (4.87 mg/dL) as compared to control (0.6 mg/dL) ($p < 0.05$). The treatment with Acid Uric 3× Tablet (0.132 mg/kg) for 21 days significantly reduced serum uric acid level (1.02 mg/dL) and xanthine oxidase activity (0.089 nM/min/mg protein) of the liver homogenate ($p < 0.05$). Whereas, allopurinol at a dose of 10 mg/kg elicited a significant reduction of serum

uric acid level (0.93 mg/dL) and xanthine oxidase activity (0.06 nM/min/mg protein) ($p < 0.05$). These results indicated that Acid Uric 3× Tablet (0.132 mg/kg) inhibited xanthine oxidase activity by 78.28% as compared to the control ($p < 0.05$). Similarly, allopurinol (10 mg/kg) inhibited xanthine oxidase activity by 85.07% at the dose of 10 mg/kg, showing slightly more potent activity than the Acid Uric 3× Tablet.

The results of the curative role of Acid Uric 3× Tablet assessed by serum uric acid level and xanthine oxidase activity of liver homogenate are given in Fig. 4B. Group-II treated with the Acid Uric 3× Tablet (0.132 mg/kg) for 7 days significantly showed a decrease in serum uric acid level (0.61 mg/dL) and xanthine oxidase activity (0.045 nM/min/mg protein) of liver homogenate ($p < 0.05$). These results showed that the administration of Acid Uric 3× Tablet (0.132 mg/kg) cured hyperuricemia in toxic group rats. Allopurinol at a dose of 10 mg/kg elicited significant reduction of serum uric acid level (0.42 mg/dL) and xanthine oxidase activity (0.043 nM/min/mg protein) of liver homogenate ($p < 0.05$).



The histopathological examination of the kidney showed glomerular damage and tubulointerstitial nephritis in potassium oxonate treated groups. Mild cellular infiltration with improvement in the renal histomorphological structure was observed in the Acid Uric 3× Tablet treated group (Fig. 5).

The anti-inflammatory activity of Acid Uric 3× Tablet was evaluated using the carrageenan-induced paw edema model. Subplantar injection of carrageenan (1%, w/v) markedly increased the paw volume of the rats, reaching its maximal at 3 h. Treatment of group-II and group-III experimental animals with Acid Uric 3× Tablet (0.132 mg/kg) and diclofenac sodium (10 mg/kg) significantly ($p < 0.05$) reduced edema volume as compared to control (Fig. 6). Edema inhibition by Acid Uric 3× Tablet and diclofenac sodium was found to be 16.12% and 20% in the first hour and was

maximum (46.42% and 46.78%) in the fourth hour of study.

DISCUSSION

Although homeopathic Acid Uric 3× Tablet is being used in curing gout in the homeopathic system of treatment, but it lacks standardization and scientific evidence against disease. Hence, Acid Uric 3× Tablet was standardized using a simple technique of RP-HPLC. The mobile phase was aqueous in nature, hence devoid of expensive ion-pairing agents and organic solvents, which have been used earlier (Dai et al., 2007). Furthermore, this detection is easy as compared to those where pre-column derivatization has been used (Chen et al., 1998). Elimination of the derivatization step makes this method suitable, less laborious and less expensive. The LOD and LOQ of the present

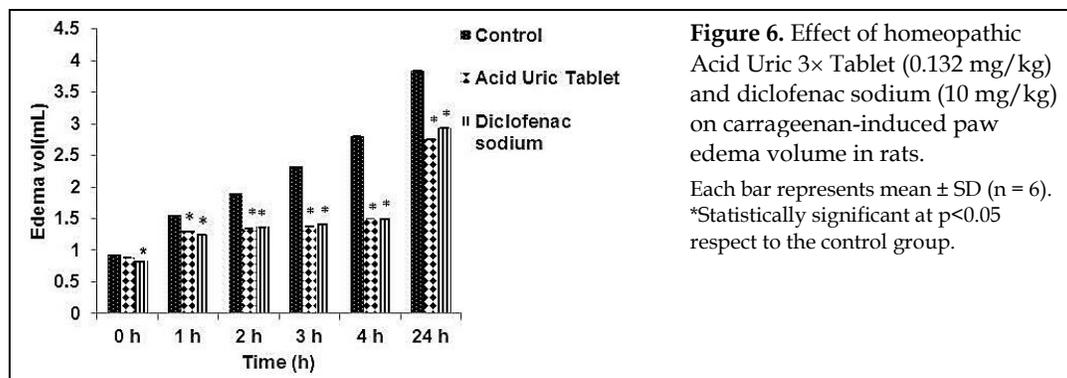


Figure 6. Effect of homeopathic Acid Uric 3× Tablet (0.132 mg/kg) and diclofenac sodium (10 mg/kg) on carrageenan-induced paw edema volume in rats.

Each bar represents mean \pm SD (n = 6).
*Statistically significant at $p < 0.05$ respect to the control group.

study were found to be lesser than the reported studies (Tanaka et al., 2013). Hence, the method can be used to quantify uric acid even at the sub-microgram level. The results of *in vitro* xanthine oxidase inhibition assay indicated that pure uric acid has no role in XO1 while lactose in concentration 1 mg/mL resulted in slight XO1. Hence, it was predicted based on the aforementioned results that Acid Uric 3× Tablet exhibits significant anti-inflammatory activity in all the *in vitro* models studied. It has been scientifically reported that during the manufacturing of homeopathic attenuation tablets by trituration, there is a change in the physicochemical characteristics of the ingredients (Kalliantas et al., 2018). Hence, the tablets under study were manufactured by a similar process, thus the probability of change in physicochemical characteristics of ingredients, which might have contributed to the significant activity of homeopathic tablets. The pharmacological evaluation demonstrated that at a dose of 0.132 mg/kg, Acid Uric 3× Tablet exhibited a significant reduction in serum uric acid levels in oxonate-induced hyperuricemia rats as compared to a positive control (allopurinol) in both protective and curative study design. Subsequent *in vivo* studies at the same dose also revealed that the remedy also remarkably inhibited hepatic xanthine oxidase activity in both study group rats. The mechanism by which Acid Uric Tablet 3× reduced serum uric acid levels is related to the inhibition of liver XO1 enzyme.

The homeopathic Acid Uric Tablet 3× also displayed notable anti-inflammatory activity in the carrageenan-induced rat hind-paw edema model. The development of carrageenan-induced edema

is a biphasic event. The initial phase (0 - 1 h) is attributed to the release of bradykinin, serotonin, histamine, and substance P. The later phase (after 1 h) is mainly due to the neutrophil infiltration into the inflammatory site and the production of large amounts of pro-inflammatory mediators such as PGE₂, IL-1 β , IL-6, IL-10 and TNF- α (Santos et al., 2012). Uric acid in physiological concentration inhibited the release of TNF α - and interleukin (IL)-1 β -induced inducible nitric oxide synthase, cyclooxygenase-2, hence inhibits the inflammatory reaction (Lai et al., 2017). The homeopathic Acid Uric 3× Tablet inhibited the edema during the acute phase of inflammation, probably by inhibiting the chemical mediators of inflammation.

CONCLUSIONS

HPLC method developed for the determination of uric acid in homeopathic Acid Uric 3× Tablet is simple, sensitive and reliable, hence may be used to standardize anti-gout remedy. This study experimentally demonstrates that Acid Uric 3× Tablet exhibits anti-hyperuricemic and anti-inflammatory activities; hence, it provides scientific evidence to its anti-gout usage.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

Contribution	Shaukat A	Hussain K	Bukhari NI	Shehzadi N
Concepts or ideas	x	x		
Design	x	x		
Definition of intellectual content	x	x	x	
Literature search	x	x	x	x
Experimental studies	x	x	x	x
Data acquisition	x	x	x	
Data analysis	x	x	x	x
Statistical analysis	x	x	x	
Manuscript preparation	x	x	x	x
Manuscript editing	x	x	x	
Manuscript review	x	x	x	x

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