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In vitro anti-gout and anti-inflammatory activity of traditionally used polyherbal anti-gout remedy

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Abstract

An anti-gout remedy comprising different parts of five herbs - seed of *Tribulus terrestris*, *Carthamus tinctorius*, *Cucumis melo*, *Punica granatum,* and seed-less fruit of *Vitis vinifera* – is being used extensively without scientific evidences to therapeutic claim. Prior investigating such claims, a well-characterized and standard formulation is required. Therefore, a standard anti-gout remedy was prepared and then investigated for *in vitro* anti-gout activity using xanthine oxidase inhibition assay and anti-inflammatory activity employing four *in vitro* models. The formulation showed dose-dependent xanthine oxidase inhibition activity (IC_{50}, 312 micrograms/mL) having linear regression equation y= 0.0782x+25.564, R^2 = 0.9909. The formulation also exhibited significant anti-inflammatory activity employing heat-induced albumin denaturation inhibition assay (IC_{50}, 1182±0.1 µg/mL) having linear regression equation y=0.0203x+26, R^2 = 0.9908, anti-proteinase assay (IC_{50}, 250±0.1 µg/mL) having linear regression equation y=0.0601x+34.943, R^2 = 0.9917, heat-induced red blood cells hemolysis inhibition assay (IC_{50}, 744±0.5 µg/mL) having linear regression equation y=0.05x+12.786, R^2 = 0.993 and hypotonicity-induced red blood cells hemolysis inhibition assay (IC_{50}, 746±0.5 µg/mL) having linear regression equation y=0.0525x+10.825, R^2 = 0.9934, respectively. The results of the current study gave scientific evidence to the folklore claim of use of polyherbal remedy in curing gout.

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Introduction

Gout is an inflammatory disease which occurs due to either higher production or impaired renal uric acid secretion or combination of both. Serum uric acid level above 6.0 mg/dL in females and above 7.0 mg/dL in males indicates the manifestation of the disease. It may occur due to some pathological condition or as a comorbidity of heart and kidney diseases or intake of certain medicines. Higher serum uric acid level may result in deposition of sodium urate crystals in joints.

This deposition results in the release of microcrystals from monosodium urate crystals which interact with monocytes, neutrophils, fibroblasts and macrophages resulting the stimulation of synthesis of proinflammatory cytokines and interleukins, responsible of acute inflammatory response and gout attack (Pouliot *et al.*, 1998; Schweyer *et al.*, 2000).

The risk of gout can be reduced either by reducing biosynthesis or increasing excretion of uric acid from the body (Umamaheswari *et al.*, 2007). Currently, different treatment modalities include xanthine oxidase inhibitors (XOI), uricosuric agents, corticosteroids and NSAID's. Allopurinol is one of the most widely used XOI (Fields *et al.*, 1996; Pacher *et al.*, 2006); however, superoxide generation leading to severe hypersensitivity reaction and severe liver function abnormalities are reported due to use of XOI (Fields *et al.*, 1996; Kong *et al.*, 2000; Umpierrez *et al.*, 1998). High cost and side effects of the modern therapies further not only restrict their use but make the reason of using several alternative remedies including folklore herbal medicines, eastern medicines (Tibbi Medicines) and homeopathic medicines, etc. It is estimated that about 80% of the World population relies on herbal medicines for their primary healthcare needs (Ekor, 2014). Herbal medicines are most commonly used alternative treatment practice worldwide and used traditionally for treating different diseases (Disch *et al.*, 2017).

Despite of the scientific usefulness of alternative and complementary medicines, scientific data and reports are limited in this aspect. Hence scientific evaluation of alternative and complementary medicines is mandatory for safety and efficacy of these therapies making it important and beneficial for public health (Fugh-Berman and Kronenberg, 2003).

A folklore polyherbal medicine comprising five ingredients (seeds of *Tribulus terrestris*, *Carthamus tinctorius*, *Cucumis melo* and *Punica granatum,* and seed-less dried fruit of *Vitis vinifera*) is being extensively used and promoted through social media in Pakistan and India for curing gout.

Though, the literature supports the use of such herbs in treating hyperuricemia, nephrolithiasis, osteoarthritis and inflammation (Arasartnum *et al.*, 2010; Arora *et al.*, 2011; Chang *et al.*, 1993; Chang and Chiang, 1995; Joshi *et al.*, 2005; Gill *et al.*, 2011; Wang *et al.*, 2004). However, we could not find any scientific evidence for effectiveness of this polyherbal remedy, which necessitates to investigate its anti-gout and anti-inflammatory potential using appropriate experimental models.

The literature contained several types of pharmacological activity models for evaluation of anti-gout and anti-inflammatory activity (Chen *et al.*, 2016; Meng *et al.*, 2014; Singh *et al.*, 2009; Martin *et al.*, 2009; Sabina and Rasool, 2008; Schiltz *et al.*, 2002). In the present study, xanthine oxidase enzyme inhibition was utilized as *in vitro* anti-gout model. Xanthine oxidase converts xanthine to uric acid; therefore, it was a specific *in vitro* model of assessing anti-gout activity.

Inflammation is relieved through various mechanisms, hence, four most commonly used *in vitro* models (heat induced egg albumin denaturation inhibition assay, proteinase inhibition assay, heat induced RBC hemolysis inhibition assay and hypotonicity induced RBC hemolysis inhibition assay) were selected in the present study. Keeping this in view, we have selected the described models to

evaluate pharmacological activity of the polyherbal remedy. The results of the current study may give scientific evidence to traditional use of the remedy.

Materials and methods

Herbs

Seed of *Tribulus terrestris*, *Carthamus tinctorius*, *Cucumis melo* and *Punica granatum*, and dried fruit of *Vitis vinifera* were obtained from the local herbal medicine market. All the ingredients were cleaned from dirt, if any, and seeds were removed from *Vitis vinifera* fruit.

Chemicals

The materials utilized in the current study were xanthine oxidase (Sigma Aldrich), xanthine (Bioworld, Biofine Plus Research Chemicals), allopurinol (Toshima, Tokyo, Japan), diclofenac sodium, hydrocortisone sodium, methanol, trypsin, casein, perchloric acid, TRIS HCl, potassium dihydrogen phosphate, sodium chloride and sodium hydroxide (Merck, Germany). Other materials included RBCs, blood from the human volunteer (who have not taken NSAIDs in the last two weeks before experiment) and in-house prepared double distilled water.

Instruments

Electronic centrifuge machine (Model-LT-LC-04R), refrigerator centrifuge machine (Sigma 2-16Kc, Germany), double beam UV-Vis spectrophotometer (Shimadzu), ultrasonicator (DSA 50-SK1-1.8L, Germany), PH meter(Hanna Instrument, Romania), thermostatic oven (U10 Memmert, Germany) and incubator (MIR-153) were used in the current study.

Preparation of anti-gout remedy

Anti-gout remedy was prepared by scaling-up the traditional method. Briefly, one hundred grams of *Tribulus terrestris* seeds, 50g seeds of each of *Carthamus tinctorius*, *Cucumis melo* and *Punica granatum,* and 50g dried seedless fruit of *Vitis vinifera* were mixed and crushed gently. The resulting mixture was divided into 7 equal parts. One part was soaked in 300mL water for 12h and then boiled for 20 min. The decoction was strained and concentrated by heating to 150mL.

In vitro anti-gout activity

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 microliters) having pH 7.5. Then, 100 microliters of each standard/sample (50 micrograms/mL), xanthine oxidase enzyme solution (0.2 units/mL) and distilled water were mixed. A control was prepared using 100 microliters of phosphate buffer instead of 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 milliliters of 1N HCl was added in 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 following equation:

$$\text{Xanthine oxidase inhibition (\%)} = (1 - \frac{\beta}{\alpha}) \times 100$$

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

In vitro anti-inflammatory activity

Egg albumin denaturation inhibition activity

Egg albumin denaturation inhibition activity was estimated following the method reported by Chandra *et al.* (2012). A test solution comprising of 4% filtered egg albumin solution (4 mL) and 1 mL of standard/sample solution (200.0 µg/mL) was incubated (37°C) for 20 min and then transferred to water bath (75°C) for 5 min. 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 contents were cooled and absorbance was determined at wavelength 660 nm using distilled water as blank. The absorbance of control and sample was used to determine percent activity in egg albumin denaturation inhibition assay using following equation:

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

Proteinase inhibition activity

The activity was determined using a method described earlier with some modifications (Leelaprakash and Dass, 2011). Reaction mixture

comprising of 1 mL of standard/sample solution (200 micrograms/mL), 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 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. 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. The absorbance of control and sample was used to determine percent activity in proteinase inhibition assay using following equation:

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

Membrane stabilization activity against heat-induced RBC hemolysis

This activity was determined following the method reported by Sakat *et al.* (2010). Blood from the healthy human (which have not taken NSAIDs since 2 weeks before experiment) was centrifuged (3000 rpm) for 10 min. RBC's separated from the blood were washed thrice using 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 (200 micrograms/mL) and 2 mL 10% RBC suspension was placed in 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. Afterwards, 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. The absorbance of control and sample was used to determine percent activity in membrane stabilization assay against heat induced RBC's using following equation:

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

Membrane stabilizing activity against hypotonicity-induced RBC hemolysis

This activity was determined following the method reported by Azeem *et al.* (2010). A reaction mixture containing 1 mL of standard/sample solution (200 micrograms/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 560nm using distilled water as blank. A control prepared replacing sample solution by hyposaline and treated like the sample. The absorbance of control and sample was used to determine percent activity in hypotonicity induced hemolysis using following equation:

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

Statistical analysis

The results were mentioned as mean ± standard deviation after analysis of all the standards/samples in triplicate. half maximal inhibitory concentration (IC50) was determined by applying linear regression on dose-response curves. The data were analyzed statistically by independent samples *t*-test using SPSS 22.0 (IBM SPSS statistics). A p value < 0.05 was regarded as significantly different.

Results and discussion

Hyperuricemia is metabolic disorder which contributes in pathogenesis of gout, nephrolithiasis, hypertension and cardiovascular complications. Hyperuricemia is characterized by elevated uric acid production; hence disorder is often controlled by reducing uric acid synthesis. Xanthine oxidase catalyze conversion of hypoxanthine to xanthine and further oxidation to uric acid. The therapeutic strategies for gout treatment include use of anti-inflammatory agents for symptomatic relief, as well as selective enzyme inhibitors in uric acid biosynthesis (Umamaheswari *et al.*, 2007). Due to some serious side effects on use of synthetic medicines, attempts have been made to find safer alternative therapy from natural source for curing gout. Hence, in current study, the traditionally used polyherbal remedy was evaluated pharmacologically using different *in vitro* models to give scientific evidences to therapeutic claim.

The results of *in vitro* anti-gout activity using xanthine oxidase inhibition model of herbal medicine and allopurinol at equivalent concentration (50 micrograms/mL) are given in Fig. 1.

The results indicated that activity of allopurinol was significantly higher as compared to herbal medicine ($p < 0.05$). Using this assay, the dose-dependent activity of the herbal medicine indicated median inhibitory concentration (IC_{50}) at 312 micrograms/mL. The linear regression equation used to determine IC_{50} was found to be y= 0.0782x+25.564, R^2 = 0. 9909 as shown in Fig. 2. These results showed that herbal medicine inhibits the oxidation of xanthine which leads to the formation of uric acid. Hence, it was experimentally demonstrated that herbal medicine exhibited significant xanthine oxidase inhibition, which was related to presence of flavonoids and phenolic compounds (chlorogenin, chlorogenic acid, ferulic acid, caffeic acid, coumarin, quercetin, kaempferol, amentoflavone and procyanidins) in the ingredients of the herbal remedy (Nile and Park, 2013; Sud'Ina *et al.*, 1993; Wang *et al.*, 2007; Al-Ali *et al.*, 2003; Mallek *et al.*, 2018; Lansky and Newman, 2007; Wang *et al.*, 2004). Inhibition of enzyme xanthine oxidase is the mechanism of action through which phenolics and flavonoids contributed to the significant biological activity of the remedy (Chang and Chiang, 1995; Chang *et al.*, 1993).

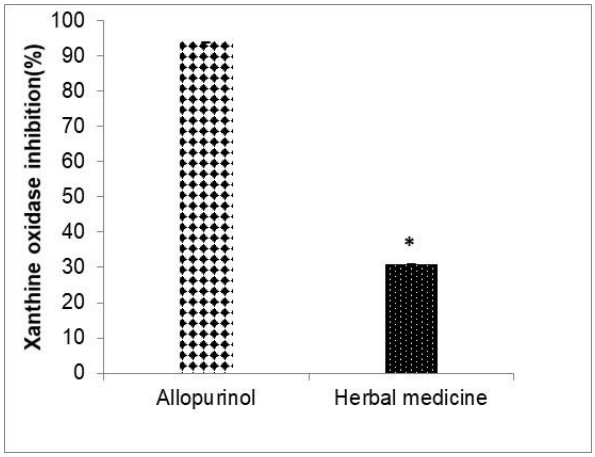

Fig. 1. Xanthine oxidase inhibition activity of herbal medicine and allopurinol at equivalent concentration (50 micrograms/mL).

The results of anti-inflammatory activity determined at equivalent concentration of sample and standard (200 micrograms/mL) using different *in vitro* models are shown in Fig. 3. In all the three models, at equivalent concentration (200 micrograms/mL), the herbal medicine exhibited 29.00% of heat-induced egg albumin denaturation inhibition as compared with 63.00% produced by diclofenac sodium, 50.00% of proteinase inhibition as compared with 77.00% produced by diclofenac sodium, 24.00% of heat induced RBC hemolysis inhibition as compared with 69.00% produced by hydrocortisone sodium and 22.00% of hypotonicity induced hemolysis inhibition as compared with 66.00% produced by hydrocortisone sodium. It effectively inhibited heat and hypotonicity induced RBC hemolysis, thus provides evidence for RBC membrane stabilization as an additional mechanism of anti-inflammatory effect (Chou, 1997).

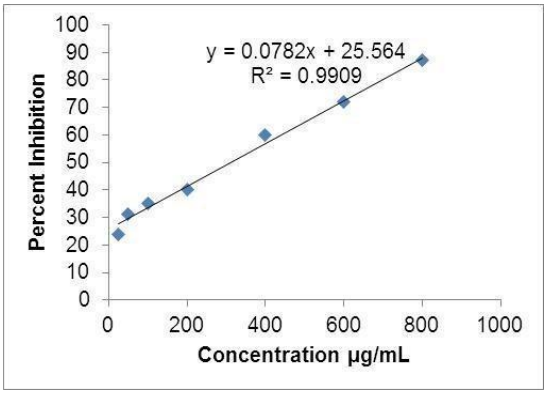

Fig. 2. Dose response curve for determination of IC_{50} in xanthine oxidase inhibition assay.

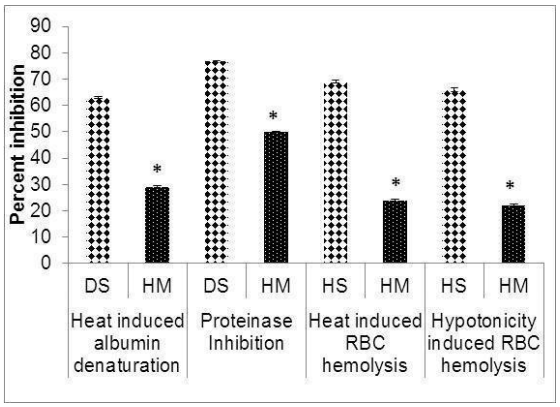

Fig. 3. Anti-inflammatory activity of herbal medicine and standard drugs at equivalent concentration (200 micrograms/mL), DS (Diclofenac sodium); HM

(herbal medicine); HS (Hydrocortisone sodium) (n=3), * significantly different than the standard ($p<0.05$).

Dose-dependent activity of herbal medicine was also determined which indicated IC_{50s} at 1182 micrograms/mL in heat-induced albumin denaturation (linear regression equation, $y=0.0203x+26$, $R^2 = 0.9908$) as shown in Fig. 4, 250 micrograms/mL in anti-proteinase activity ($y=0.0601x+34.943$, $R^2 = 0.9917$) as shown in Fig. 5, 744 micrograms/mL in heat-induced RBC hemolysis inhibition assay ($y=0.05x+12.786$, $R^2 = 0.993$) as shown in Fig. 6 and 746 micrograms/mL in hypotonicity-induced RBC hemolysis inhibition assay ($y=0.0525x+10.825$, $R^2 = 0.9934$) as shown in Fig. 7.

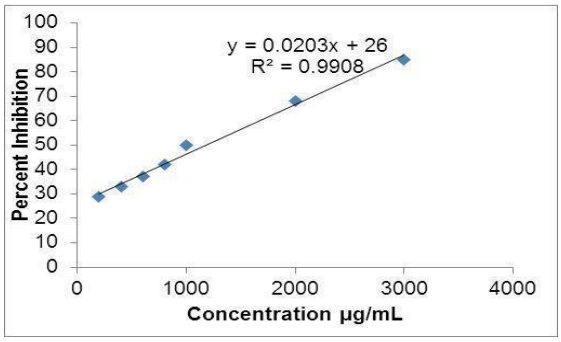

Fig. 4. Dose response curve for determination of IC_{50} in heat induced egg albumin denaturation inhibition assay.

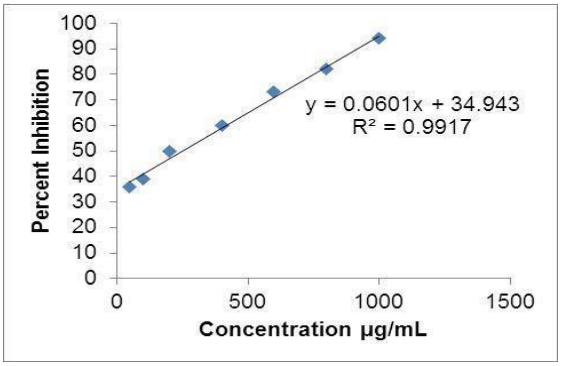

Fig. 5. Dose response curve for determination of IC_{50} in proteinase inhibition assay.

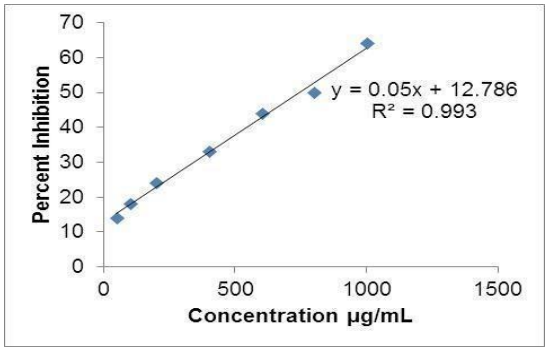

Fig. 6. Dose response curve for determination of IC_{50} in heat induced RBC hemolysis inhibition assay.

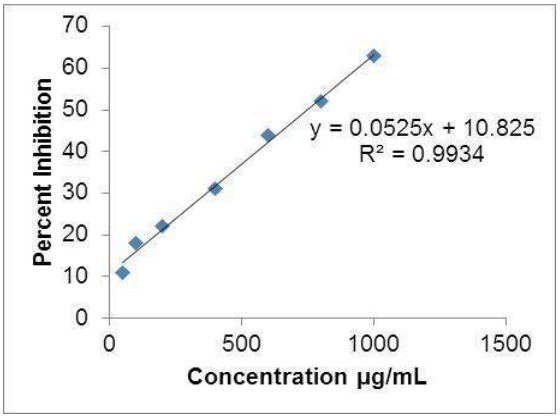

Fig. 7. Dose response curve for determination of IC_{50} in hypotonicity induced RBC hemolysis inhibition assay.

Most common phenolic compounds (ferulic acid, chlorogenic acid and caffeic acid) and flavonoids in the ingredients of the remedy exhibit significant anti-inflammatory activity (Chao *et al.*, 2009; Cunha *et al.*, 2016; Lim *et al.*, 2008). Hence significant anti-gout and anti-inflammatory activity of the traditionally used polyherbal remedy was attributed to the presence of these pharmacologically active phytochemical compounds.

Conclusion

This study experimentally demonstrates that polyherbal anti-gout medicine exhibits anti-hyperuricemic and anti-inflammatory activities as indicated by results of different *in vitro* models which was attributed due to presence of different phytochemicals in the ingredients of the polyherbal remedy. Hence, findings of the present study gave scientific evidence for the traditional use of herbal remedy for treating gout.

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