ORIGINAL ARTICLE

Antihyperuricemic and xanthine oxidase inhibitory activities of Silymarin in a rat gout model

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Abstract

Introduction: Gout is a common metabolic defect spread around the world. It characterized by hyperuricemia, which resulting from the prolonged rise of uric acid (UA) levels in the blood, leading to increase the deposition of urate crystals in the joints and kidneys. The present study performed to investigate the efficacy of silymarin as antihyperuricemic agent. **Materials and Methods:** Enzyme assay was done using bovine milk xanthine oxidase (XO). The XO inhibitory activity *in vitro* was carried out using different doses of silymarin, and the degree of XO inhibition (XOI) was expressed as IC_{50}. The antihyperuricemic of silymarin was investigated in the potassium oxonate-induced hyperuricemic rat model for 7 consecutive days of oral treatment of 10, 25, and 50 mg/kg doses. **Results:** The study results revealed that the silymarin has a potent activity of XOI with IC_{50} = 5.84 µg/mL as compared to standard drug, allopurinol IC_{50} = 1.85 µg/mL. In addition, these results showed that all doses of silymarin were able to be significant reduced serum UA levels in the hyperuricemic rats. **Conclusion:** Silymarin showed a significant effect on lowering the level of UA in the evaluated model, and therefore, it may be a promising agent for treating gout because of the possession of an antihyperuricemic effect through the inhibitory activity of xanthine oxide.

Key words: Antihyperuricemic, gout, silymarin, xanthine oxidase

INTRODUCTION

Uric acid (UA) is the insoluble final product of purine digestion (DNA, RNA, and nucleotides). In the human body, nearly two-thirds of UA amount are the result of the degradation of endogenous purines, while the rest of the diet. Hyperuricemia means the precipitation of UA inside and around the joints and other tissues as a monosodium urate (MSU) crystal, and shedding of crystals into the synovial fluid generates a local inflammatory reaction. This caused joint inflammatory arthritis is termed gout. Gout is typically to a large degree painful, conventional therapy is nonsteroidal anti-inflammatory drug as a first remedy,[1,2] urate-lowering drugs such as allopurinol and probenecid.[3] Hyperuricemia is elevated in people with renal dysfunction, cardiovascular disease,[4] and hypertension.[5]

UA level in blood, furthermore, definitely increase with metabolic syndrome such as obesity, dyslipidemia, hyperglycemia, and insulin resistance.[6] UA was consumed as endogenous antioxidant for potent scavenger of reactive oxygen species and hydroxyl free radicals (OH). It is react with peroxy nitrile and stops nitric oxide synthase after was common believed it is metabolically inactive material, so UA acts as a pro-inflammatory and pro-antioxidant factor.[6,7] UA in blood indicator of pathologic circumstances damage by oxidation such as liver harm, hyperlipidemia, atherosclerosis, chronic heart failure, diabetes,[8] renal injury, fibrosis, and stimulating vascular smooth muscle proliferation.[9]

Silymarin is flavonoids compounds exist in *Silybum marianum*, as a chemical mixture of four isomers; silibin (major isomer), isosilbin, silycristin, and silydianin. This drug is an effective liver protective agent because it has a

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positive effect on metabolism and organ function in liver cells, affecting its ability to regenerate due to two major processes: Antioxidant activity and protein recovery. The drug also prevents the work of toxics and xenobiotics from penetrating the liver by stabilizing the hepatocytes membranes.[10,11]

Most of the biological activities of silymarin are attributed to the silibinin isomer, the main (60–70% of isomers content) and most effective ingredient.[11] Many different studies in both animal and human showed that silymarin has a good safety profile and safe even when given at high doses (>1500 mg/day).[12] Silymarin is very useful in the treatment of jaundice patients by facilitating the conjugation of bilirubin with glucuronic acid or through the inhibition of ϰ-glucuronidase enzyme that produced from the toxic pathogenic bacteria in the intestine.[13]

Silymarin demonstrations good hepatocytes protective and antioxidant potential against diethyl nitrosamine prompted hepatocellular injury,[14] silymarin declines the poisonousness induced by gold nanoparticle in diabetic rats.[15]

Antioxidant activity of silymarin increases the concentration each of glutathione, catalase, and superoxide dismutase, which are main antioxidant in human body that detoxifies of drugs and chemicals. It has potent antioxidant effect resulted from trapping free radicals, superoxide, and peroxide radicals that are produced by lipid oxidation process.[16,17]

The aim of this study is to investigate the antihyperuricemic activity of silymarin flavonoids in the potassium oxonate-induced hyperuricemic rats. Further, the present study also aimed to prove the inhibitory activity of silymarin against xanthine oxidase (XO) for determining the mechanism action of UA -lowering effect of silymarin.

MATERIALS AND METHODS

Reagents and Kits

UA kit was purchased from Biolabs Company (Maizy, France). XO from bovine milk (Grade I), xanthine and allopurinol were provided from Sigma-Aldrich (Dorset, England). All other chemicals were supplied from Merck (Darmstadt, Germany). All chemicals and reagents used in this study were of analytical grade.

Animals

This study included using (36) male Wistar rats (150–170 g) supplied from the unit of animals' house at College of Pharmacy, Basrah University. Both rats and mice were separated into different groups (n = 6), then the animals were accommodated in isolated plastic cages and kept in the animal's room under a regulated condition at temperature 25 ± 2°C and humidity 30 ± 15% with 12-h dark/12-h light cycle for a week before being used for acclimatization. They were fed a standard chow and water *ad libitum*. Animal Ethics Committee, University of Basrah, Iraq (no.2013/32), authorized all dealing procedures with animals that described in this study.

In vitro XO Inhibitory Activity

The XO inhibitory effect of silymarin acid was assessed spectrophotometrically at 290 nm according to Sunarni *et al*.[18] and Yumita *et al*.[19] with minor changes. The mixture assay consists of 0.9 mL of 0.05 M sodium phosphate buffer (pH 7.5 at 25°C), 1 mL of silymarin solution (100 µg/mL in DMSO), and 0.1 mL of XO enzyme solution (0.1 unit/mL in phosphate buffer, pH 7.5) was prepared in cold buffer directly before using. After a 15-min preincubation at 25°C, the reaction was started by addition of 2000 µL of freshly prepared solution of substrate (0.15 mM xanthine solution). Next, a further incubation process was achieved for the reaction mixture at 25°C for 30 min. After addition of 1 mL of 1 N HCl solution into assay mixture for stopping the reaction, the absorbance was recorded at wavelength 290 nm using UV/vis spectrophotometer against the blank which is prepared in the same procedure but with replacement of enzyme solution by phosphate buffer. The positive control solution was prepared using allopurinol (100 µg/mL) in DMSO. The inhibitory activity of XO was established as the inhibition percentage (%):

$$\% \text{ XO inhibition (XOI)} = (1-\alpha/\beta) \times 100$$

Where, α is the activity of XO without tested substance (silymarin) and β is the activity of XO with the presence of silymarin.

Different concentrations of both silymarin and allopurinol (100, 50, 25, 10, 5, 4, 3, 2, and 1 µg/mL) were used for the evaluation of XO inhibitory activities. Then, the dose–response logarithmic curve was applied to find the median maximum inhibitory concentration IC_{50}.

Drug Administration

Allopurinol and silymarin were suspended in 0.5% sodium salt of carboxymethylcellulose (CMC) and CMC-Na (vehicle). Potassium oxonate (250 mg/kg), indomethacin (3 mg/kg), and MSU crystals (40 mg/mL) were suspended in 0.9% sterile saline. All solutions were prepared freshly before use for *in vivo* experiments.

Evaluation of Antihyperuricemic Activity

The antihyperuricemic activity of α-lipoic acid was investigated using the potassium oxonate-induced hyperuricemia in the

rat's model according to Haidari *et al*.[20] and Nguyen *et al*.[21] with changes. Animals were fasted by withdrawing of food and water 2 h before drugs administration. Experimental animals (rats) were divided randomly into six groups (n = 6). The uricase inhibitor (potassium oxonate) at a dose of 250 mg/kg was injected intraperitoneally (i.p.) to rats of groups (2–6) in the 1st, 3rd, and 7th days of the experiment period. Rat's groups were administered with oral treatments of the vehicle, allopurinol and silymarin solutions by oral gavage 1 h after the administration of potassium oxonate, once a day for 7 consecutive days of experiment. Animals of normal control (Group 1) and hyperuricemic control (Group 2) were received only vehicle through oral administration. Standard drug group (Group 3) was treated orally with allopurinol (10 mg/kg). Sample Groups 4–6 were treated orally once a day with silymarin at the doses of 10, 25, and 50 mg/kg, respectively, throughout the days of the experiment. Whole blood samples were collected from each rat by cutting tail vein 2 h after last administration of tested drugs. The blood was permitted to clot for 0.5 h at room temperature and then centrifuged at 3500 rpm for 5 min to get the serum. The sera were stored at −20°C until the UA is assayed.

UA Assay

The enzymatic colorimetric method was employed to determine the serum UA levels using a standard diagnostic kit (BioLab, France).

Statistical Analysis

The results of all trials in this study are stated as mean ± standard error mean. Statistical analysis was carried out by one-way (ANOVA) pursued by the Dennett's t-test. The values of $P < 0.05$ were considered as statistically significant.

RESULTS

In vitro XO Inhibitory Activity

The inhibitory effects of silymarin and allopurinol for bovine milk XO at different concentrations represented in Table 1. Each has revealed more than 50% of XOI at the concentration of 4 µg/mL. At highest concentration of 100 µg/mL, the silymarin resulted in 81% of XOI activity, while the standard XO inhibitor, allopurinol demonstrated 95% of XOI activity at the same concentration. The XO inhibitory effects for both silymarin acid and allopurinol also stated in the term of IC_{50}, which is represented the concentration of standard drug or tested sample that is required for 50% inhibition of XO activity under the same experimental conditions. The IC_{50} values were calculated according to the dose–response logarithmic curve using GraphPad Prism V 6.05 program (GraphPad Prism software, Inc., USA), where the value was equal to 1.85 µg/mL for allopurinol and 5.84 µg/mL for silymarin, respectively, as shown in Figure 1.

Antihyperuricemic Activity

To assess the existence of antihyperuricemic effect of the silymarin, the potassium oxonate-induced hyperuricemia model in rats used in this study. As shown in Table 2, the i.p. injection of uricase inhibitor, potassium oxonate (250 mg/kg) obviously increased the serum UA levels in rats compared with healthy normal control group. The administration of

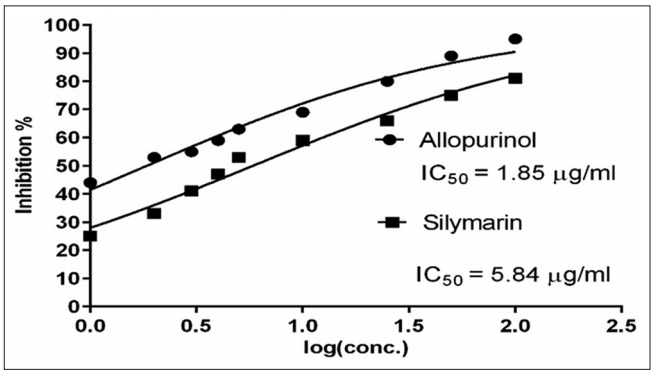

Figure 1: Xanthine oxidase inhibitory activity and IC_{50} values of silymarin and allopurinol

Table 1: Xanthine oxidase inhibitory activity of silymarin and allopurinol at different concentrations

Concentration (µg/mL)	XO inhibitory activity (%) of	
	Allopurinol	Silymarin
100	95±1.5	81±2.2
50	89±0.6	75±0.7
25	80±1.1	66±1.8
10	73±2.1	59±1.4
5	67±2.0	53±0.3
4	61±0.4	47±0.2
3	55±0.6	41±1.1
2	53±1.6	33±0.8
1	44±1.2	26±0.7

Table 2: Effects of allopurinol and silymarin on the serum UA levels in the normal and potassium oxonate-induced hyperuricemic rats

Group	Dose (mg/kg)	n	Serum UA (mg/dL)
Normal control	-	6	1.2±0.2
Hyperuricemic control	-	6	4.6±0.7
Standard drug (allopurinol)	10	6	1.5±0.6***
Test (silymarin)	10	6	2.6±0.4***
	25	6	2.4±0.5***
	50	6	2.0±0.3***

Each value is the mean±SEM, *P<0.05, **P<0.01, ***P<0.001 compared with hyperuricemic control. Data were analyzed using one-way ANOVA followed by Dennett's test

standard XO inhibitor, allopurinol (10 mg/kg, p.o), was able to significant lower ($P < 0.001$) the serum UA levels of hyperuricemic rats (positive control group) to values close of normal control. The consecutive 7-day treatment of rats with silymarin at the doses of 10, 25, and 50 mg/kg significantly reduces ($P < 0.001$) the serum UA levels as compared with hyperuricemic control group in all doses above.

DISCUSSION

Any hyperuricemia case needs to inhibit the XO enzyme. The first inhibitor was allopurinol and still used to this date. XO is metallic enzyme contained molybdenum (Mo) metal, the enzyme deprotonating xanthine and oxidized it to UA. The silymarin flavonoids may be coordinate with molybdenum of enzyme and/or bind with Mo-OH group in an active site of the enzyme and inhibit the reaction of UA formation. Silymarin can reduce the Mo center of the enzyme led to inhibit the XO.[22]

Although the allopurinol gave activity more than the silymarin to reduced UA level, both of them are award significant activity ($P < 0.001$) to lowering UA level. The silymarin safe dose can increase up to 1500 mg/day, but allopurinol LD_{50} oral > 500 mg/kg (rat), in mouse is 78 mg/kg. The oral toxic dose low TDLo for allopurinol in rats is 10 mg/kg and in mice is 100 mg/kg.[23] Hence, as a final result, we can use silymarin in high dose, but allopurinol is not, this will give advantage to use silymarin as more active than allopurinol which is toxic substance cannot give in a high dose.

XOI activity of plants was interrelated with their completely phenolic compounds contents. The flavonoids structures-activity affects XOI through interaction with the molecular target of flavonoids. The selection of flavonoids as effective XO inhibitors requires an existence hydroxyl group in C-5 and C-7, as well as a double bond between C-2 and C-3 or a planar structure of flavones. The substitution of hydroxyl groups at C-3 and C-7 of some flavonoids by glycosylation or methylation leads to low inhibitory activities of XO. Flavonoids with substituted OH group of some specific positions obstruct binding with the enzyme active site that leads to decrease inhibitor activity of the compounds.[24] Silymarin also expands total antioxidant ability, suppresses destructive oxygen free radicals, and prevents oxidative stress destruction.[25] Modern researches are interested attentive on searching for more effective and safer agents for gout from medicinal plants. Wong *et al*.[26] stated that the mechanisms of antigout effect by the antioxidants are needed to be established in future studies. Silymarin compounds play an important role in the defense of human cells from damage by free radicals through its antioxidant activity.[27] The XO inhibitory activity of glycosides is low as compared with its aglycon part because of competition on the binding site of xanthine in XO enzyme.[28]

The current finding was consistent with other researchers suggesting that potential synergies between silymarin constituents contribute to their overall antioxidant activity as an effective antihyperuricemic agent.

Many studies have found that XO is controlled in many cardiovascular conditions such as myocardial ischemia and heart failure associated with improved oxidative stress, so silymarin prevents XO equivalence with an antioxidant activity that is very useful. In addition, the high level of UA is associated with coronary artery disease. Thus, reducing the composition of free radicals is an effective approach to reduce the level of UA, together control of cardiovascular disease.[29]

CONCLUSION

The current study has revealed that the silymarin may be a potential alternative phytotherapy in the treatment of gouty patients due to the reduction of UA synthesis through the inhibition of XO activity.

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