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Novel use of *Citrullus colocynthis* Cortex Extracts for Gout Treatment.

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ABSTRACT

The present study was aimed to investigate the effect of methanolic extracts of *citrullus colocynthis* cortex prepared from used part, on xanthaine oxidase activity, which tested *in vitro*, at 100 µg/mL concentration **for their** inhibition potencies expressed as % inhibition of XO activity. The test plant was found hypouricaemic activity *in vivo* also with inhibition % of xanthine oxidase about (78.30±0.92) compared with Allopurinol with significant (p≤0.05). The fraction of these species was used in traditional medicinal system for the treatment of gout, rheumatism, and residual fractions at a dose of 0.5 mg/kg body weight. , and its effect appear by reduce the serum urate level and inhibitory actions on the XO enzyme activities in the mouse liver. Phytochemical screening of the cortex of *citrullus colocynthis* revealed the presence of tannins, flavonoids, alkaloids and terpenoids. The presence of phytochemical constituents may be partly responsible for the beneficial effect of the fractions on hyperuricaemia and gout. These results suggest that the fraction of *citrullus colocynthis* could be used as a potential source to treat gout and other inflammatory disorders.

Keywords: *citrullus colocynthis,* gout, hyperuricaemia, uric acid, xanthine oxidase, oxonate.

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INTRODUCTION

Xanthine oxidoreductase (XOR) is a highly versatile enzyme that is widely distributed among species (from bacteria to man) and among the various tissues of mammals. It is a member of the group of enzymes known as molybdenum-iron-sulfur-flavin hydroxylases. XOR has two interconvertible forms, xanthine oxidase (XO) and xanthine dehydrogenase (XDH). Both the enzymes catalyze the oxidation of hypoxanthine to xanthine and then to uric acid, the final reactions in the metabolism of purine bases [1]. There is substantial evidence that over activity of this enzyme leads to a condition, generally called as gout [2].

Gout is one of the most common metabolic disorders with a worldwide distribution and continues to be a major health problem. It affects around 13% of the male population and 5% of the female population [3]. Gout is characterized by an excessive concentration of uric acid in the blood , causing the accumulation of monosodium urate crystals in the joints and kidneys leading to acute gouty arthritis, tophi of the joints and extremities, and uric acid nephrolithiasis [4]. Elevated levels of uric acid not only leads to gout, but also results in the development of hypertension, cardiovascular disorders, diabetes, obesity, hyperlipidemia and cancer [5]. The therapeutic approach to treat gout is to use either uricosuric agents or xanthine oxidase inhibitors (XOI). XOIs block the synthesis of uric acid from purines and they are much useful when compared to other drugs, since they possess lesser side effects. Allopurinol remains to be the dominant clinically used xanthine oxidase inhibitor, however adverse effects limits its therapy [6]. Thus, there is a need to develop compounds with XOI activity which is devoid of the undesirable side effects of allopurinol. A potential source of such compounds can be obtained from medicinal plants [7,8].

Citrullus colocynthis (L.) Schrad. (Cucurbitaceae), commonly known as bitter-apple, colocynth, or wild-gourd, is a tropical plant that grows abundantly in many place in the world. The cortex of *C.colocynthis* have been subjected to a range of pharmacological, phytochemical and nutritional investigations in recent years. It has been shown to contain 17% of a fixed oil with high proportion of unsaturated fatty acids, mainly linoleic acid (60-70%), oleic acid (11.7-15%) and a very low n-3 poly-unsaturated FA level (0.5%). It is also rich in antioxidant eg. tocopherol, polyphenol and plant sterol [9].

MATERIALS AND METHODS

PLANT MATERIAL

The plant material consists of dried powdered prepared as below of the cortex *Citrullus colocynthis* belonging to the family Cucurbitaceae collected from Iraqi desert.

PREPARATION OF THE EXTRACT AND FRACTIONATION

The plant was soaked in water, washed to get rid of any adhering dust and impurities, and then oven dried at 40ºC for 72 hours. The dried were ground to fine powder using mill and pass through 24 mesh sieve to generate a homogenous powder. The finely powdered plant are kept in a dark place at room temperature until the time of use. About 1g of the dried powdered was soaked with 50 ml of methanol: water (7:3) for 48 hrs was incubated in room temperature .The extract was filtered using Whatman No.1 filter paper, the solution was evaporated by rotary evaporator at 40 ºC .All extract were kept in vacuum desiccators over anhydrous calcium chloride and were kept in the fridge at 4 ºC to be test.

Phenolic Compound Analysis

Determination of Total Phenolic Content

Total phenolic content in extract were estimated by Folin-Ciocalteau reagent as desicribed by [10].Then samples were analyzed by High Performance Liquid Chromatography (HPLC) system, model Shimadzu 10AV-LC equipped with binary delivery pump model LC-10AV, the eluted peaks were monitored by UV-VIS10A-SPD spectrophotometer. Standards of suspected compound were run similarly for identification and quantification ,concentration of each isolated compound.

The fractions containing low-molecular weight phenolic compounds(5ml) were collected using a fraction collector and their absorbance was measured at 278 nm. The fractions were eluted from the column with acetonitrile-water (80:20; v/v) [11]. The elutes then pooled into major fractions. Organic solvents were evaporated at 45°C using rotary evaporator and the water solution of the fractions was lyophilized [12].

Determination of Lethal Dose (LD_{50}):- LD_{50} conducting by formula reported in [13].

Xanthine Oxidase Inhibitory Activity Assay

The inhibitory effect on XO was measured spectrophotometrically at 295 nm under aerobic condition, with some modifications, following the method reported by [14]. A well known XOI, allopurinol (100 µg/ml) was used as a positive control for the inhibition test. The reaction mixture consisted of 300 µl of 50 mM sodium phosphate buffer (pH 7.5), 100 µl of coumarin fractions solution dissolved in distilled water or DMSO, 100 µl of freshly prepared enzyme solution (0.2 units/ml of xanthine oxidase in phosphate buffer) and 100 µl of distilled water. The assay mixture was pre-incubated at 37°C for 15 min. Then, 200 µl of substrate solution (0.15 mM of xanthine) was added into the mixture. The mixture was incubated at 37°C for 30 min. Next, the reaction was stopped with the addition of 200 µl of 0.5 M HCl. The absorbance was measured using UV/VIS spectrophotometer against a blank prepared in the same way but the enzyme solution was replaced with the phosphate buffer. Another reaction mixture was prepared (control) having 100 µl of DMSO instead of test compounds in order to have maximum uric acid formation.

The inhibition percentage of xanthine oxidase activity was calculated according to the formula = $(A_{control} - A_{sample}) / A_{control} \times 100\%$ [15].

Laboratory Animals

Albino Swiss male mice were obtained from National Center for Drug Control and research / Ministry of Health / Baghdad with ethics agreement (8/ 2014). Their age at the start of experiments was 6-8 weeks, and their weight 28±1.5 gram. They were divided into groups , and each group was kept in a separate plastic cage (details of these group are described below). The animals were maintained at a temperature of 23-25 ºC and they had free excess to food (standard pellets) and water.

Animal Model of Hyperuricemia in Mice

Experimentally-induced hyperuricemia in mice (due to inhibition of uricase with potassium oxonate) was used to study anti-hyperuricemia and antioxidant effects of coumarin [16]. Briefly, 150 mg/kg potassium oxonate (PO) dissolved in 0.9% saline solution was administrated intra-peritoneal to animal 1 hour before administration of test compound.

Experimental Design

Mice were randomly divided into four groups (6 mice per group). In group 1, the normal group, each animal received only water as vehicle .Group 2, the hyperuricemia group, PO (150 mg/kg) was administrated intra-peritoneal. In group 3, each animal was first injected intra-peritoneal the same dose of PO 1 hour before administration of test compound and after 3 hour received 0.5 mg/kg fraction of *citrullus colocynthis* extract. The group 4, each animal was first injected intra-peritoneal the same dose of PO 1 hour before administration of test compound and after 3 hour received 10 mg/kg Allopurinol.

Sample Preparation

Blood sample was taken from each mice, mice were anesthetized with ether and decapitated. The blood was allowed to clot for 1 hour at room temperature and the centrifuged at 3500×g for 5 min to obtain serum. The serum was stored at **-20 C ^{0}** for future laboratory measurements.

Determination of Serum Uric Acid

Uricase acts on uric acid to produce allantion, carbon dioxide and hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase reacts with a chromogen (amino-antipyrine and dichloro-hydroxybenzen sulfnate) to yield quinoneimine, a red colored complex. The absorbance measured at 520 nm [17].

Acute Toxicity Studies

Albino mice maintained under standard laboratory conditions was used. which divided into five groups consisting of 6 each; the animals received dose (1,1.5,2,2.5,5 mg/kg, body weight) of coumarin. Animals were kept overnight fasting prior to drug administration. After the administration of the coumarin fraction, food was with held for further 3-4 hours. Animals were observed individually at least once during the first 30 minutes after dosing, once daily cage side observations included changes in skin and fur, eyes, also respiratory rate, circulatory (heart rate), and CNS (ptosis, drowsiness, gait, and convulsions) changes [18].

Histopathological Procedure

Livers tissues was excised and immediately fixed in 10% formalin for 24-48 followed by dehydration of samples by increasing concentrations of ethanol solution (70%, 80%, 90%, and 95% for 2 hours in each concentration and 4 hours divided in two changes of 100%). Clearing by 2 changes of xylene was done for 15 minutes for each one, then embedding of samples in paraffin wax. Thin sections (5-6 micron) of the paraffin embedded blocks were taken and dewaxed in xylene for 6 minutes and hydrated by decreasing concentrations of ethanol (100%, 95%, 90%, 80%, and 70%) followed by distilled water for 2 minutes. Then, these sections were stained with haematoxylin for 2-5 minutes, washed in running tap water for 2-3 mins, followed by removal of excess stain by 0.5-1 % HCL in 70% alcohol for few seconds, washed in running tap water for at least 5 minutes, then stained with eosin for 1-2 minutes. Sections were washed in tap water, dehydrated through increasing concentrations of ethanol, cleared in xylene, and finally mounted with DPX to be transferred for histopathological examination under light microscope [19].

Statistical Analysis

Data obtained were expressed as mean ± standard deviation and statistically analyzed to verify the accuracy and sensitivity of the measurements. The protocol for the statistical analysis applied throughout the experimental part by *SPSS* version 17 by using t test. The probability (P) of the measurements was considered to be significant (at < 0.05).

RESULT AND DISCUSSION

Total Phenolic Contents in Extract Plant

Table (1) showed the total phenolic as mg gallic acid equivalent / 100 mg dry weight of cortex *Citrullus colocynthis*.

Table 1: Total phenolic contents of plants under investigation

Extract of plant	Total phenolic (mg gallic acid equivalent / 100 g)
cortex*Citrulluscolocynthis*	47.66 ± 0.83

Phenolic Compound Ananlysis

Results obtained by HPLC showed that coumarin has the major concentration (156.6 µg/ml) than other compounds. So all following results represent the effect of coumarin on different parameters.

***In Vitro* Xanthine Oxidase Inhibitory (XOI) Activity**

The result shows that the highest XO activity was shown by 70% methanol extract of cortex *Citrullus colocynthis* (coumarin fraction) with 78.30±0.92 compared with positive control (Allopurinol) ($P \leq 0.05$) as show in the table (2).

Table 2: Xanthine oxidase inhibitory activity of coumarin fraction compered with positive control (Allopurinol)

Extract of plant	Fraction	% xanthine oxidase inhibition
1-*citrullus colocythis*	coumarin	78.30±0.92*
2-allopurinol (positive control)		69.33±4.04

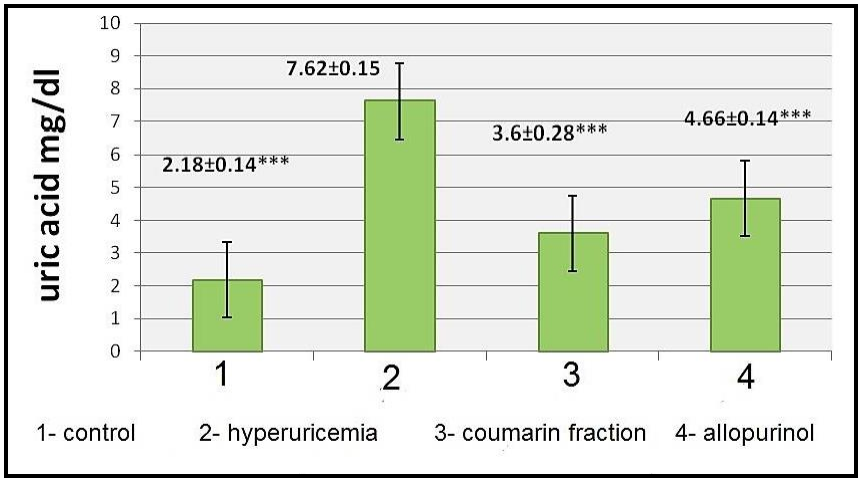
Result are expressed as mean ±SD(n=3) *indicates p≤0.05

Phytochemical screening of the plant revealed the presence of flavonoids, phenolics, saponins and triterpenoids accounting for its antioxidant property. Flavonoids are a group of polyphenolic compounds which exhibit several biological effects such as anti-inflammatory, anti-hepatotoxic, anti ulcer activities, etc. They have also been reported to inhibit the enzyme xanthine oxidase [20].

Effect of Coumarin Fraction of *Citrullus Colocynthis* on Serum Urate Level in Mice

Administration of the uricase inhibitor, potassium oxonate resulted in significant ($P<0.001$) hyperuricaemic in mice, as indicated by an increase in the serum uric acid levels when compared to the control group. Pre-treatment with the coumarin fractions of *Citrullus colocynthis* for three days significantly ($P<0.001$) reduced the serum urate levels, when compared with the hyperuricaemic control group. The standard drug allopurinol at a dose of 10 mg/kg elicited significant ($P<0.001$)reduction of serum urate level compared to hyperuricaemic mice (figure 1).

Figure 1: Effect of the coumarin fraction of *Citrullus colocynthis* on serum urate levels in control and experimental animals.



These results suggest that coumarin fractions were capable of reducing the accumulation of purine metabolites in blood following oxonate induction. Our unpublished data suggests that the fraction has highest phenolic and flavonoid content as determined by pyrocatechol and quercetin equivalents respectively. In conclusion, the data reported in the present study indicates that coumarin fraction of *citrullus colocynthis* have significantly reduced the serum urate levels in hyperuricaemic animals. This may be due to the inhibition of XO activity and the presence of phytochemical constituents.

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Determination of Lethal Dose LD_{50}

Results obtained in (mg/kg) can be classified according to the potential acute toxicity of the test compound into several classes, from extraordinary or less toxic 1mg/kg up to a relatively less harmful 5000-15000 mg/kg .In this study, we found LD_{50} equal 1.72 mg/kg as shown in table (3).

The result can serve as a guide in selection of doses for pharmacological studies. The acute toxicity test is mild and it suggests that the coumarin are safe for use. The results support the traditional use of coumarin as anti gout and inflammatory conditions and suggest the presence of biological active of coumarin which may be worth further investigation and elucidation.

Table 3: Effect of different dose of coumarin fraction of *citrulluscolocynthis*

Groups	Dose mg/kg	Numberof animal	Number of died animal	Mean	Product
control	0	6			
Treatment1	0.5	6	0		
Treatment2	1	6	1	0.5	0.25
Treatment3	1.5	6	3	2	1
Treatment4	2	6	5	4	2
Treatment5	2.5	6	7	6	6.25

Table 4: Important changes in behavior animals during 24 hour from administration different dose of coumarin fraction of *citrulluscolocynthis*

Observation	Motor activity	Anorexin	Abnormal gait on toes	Twitches	Palpebral potosis
Control mg/kg	-	-	-	-	-
Treatment1 0.5mg/kg	-	-	-	-	-
Treatment2 1mg/kg	+	+	+	-	+
Treatment3 1.5mg/kg	++	++	++	+	+
Treatment4 2mg/kg	+++	+++	+++	++	++
Treatment5 2.5mg/kg	+++	+++	+++	+++	+++

Legend: - = Absent, + = Trace, ++ = Moderate, +++ = Abundance

Histopathological Findings

Liver Histology

Free radical formation during the metabolism of oxonate by hepatic microsome, cause lipid peroxidation of the cellular membrane leading to the acute inflammatory of hepatocytes. Mice treated with oxonate developed significant hepatic damage as compared to controls (Figure 2a and b). An ameliorative effect was obtained in hyperuricemic mice by oral treatment with either allopurinol (Figure 2d) or coumarin fraction of *citrullus colocynthis.* (Figure 2c). Histopathological data substantiate liver dysfunction. There are inflammatory leucocytic infiltrations considered, [21], as a prominent response of the body tissue facing injurious impacts. The intrahepatic blood vessels, central and portal veins are congested and their lining epithelia are eroded. Nevertheless, mice treated with oxonate developed significant hepatic damage as compared to controls. An ameliorative effect was obtained in hyperuricemic mice by treatment with coumarin fraction of *citrullus colocynthis* extract.

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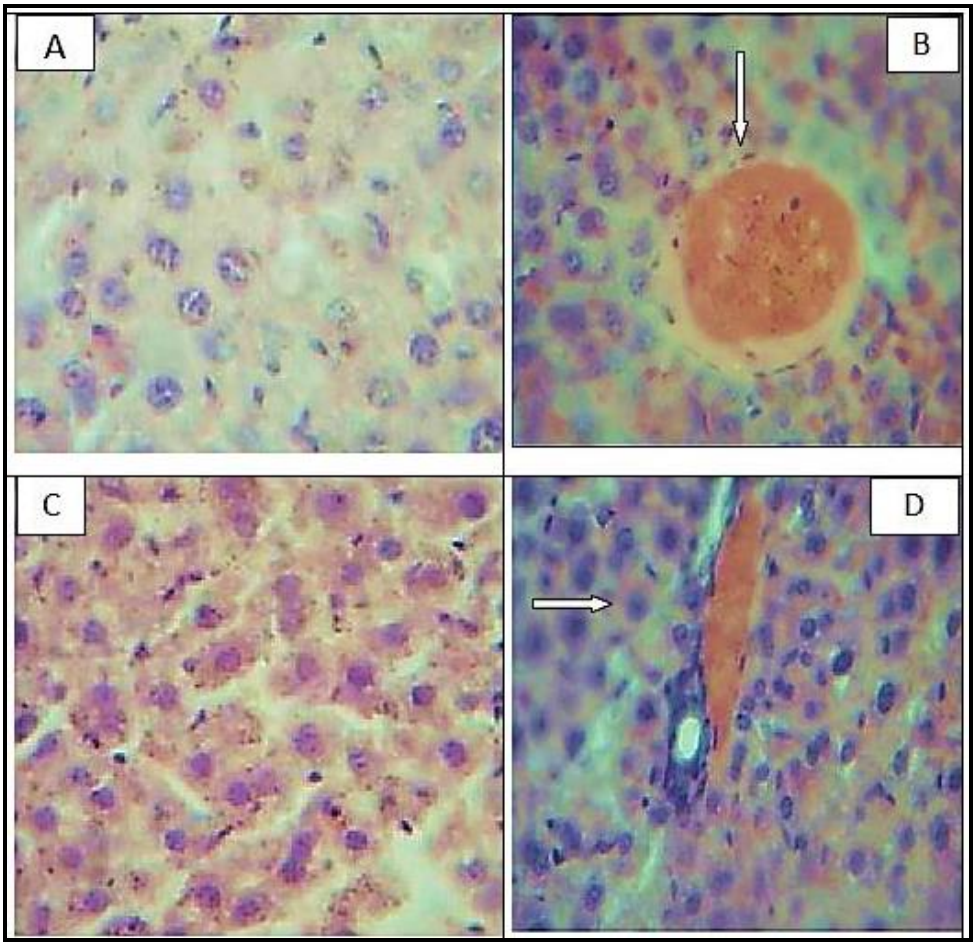
Fig 2: Microscopic observation of mice liver



Result are expressed as mean ±SD (n=3) ***indicates p≤0.001

(A) Represent section from control depicting the normal structure of lobule and hepatocytes; (B) section from the oxonate showing the acute inflammatry in hepatocytes; (C) Section from the fraction of *citrulluscolocynthis*extract showing the architecture of the lobule and hepatocytes; (D) section from the allopurinol (10 mg/kg bw) group showing low inflammatory in hepatocytes; Filled circle: normal hepatocyte, open circle: hyper vacuolation of hepatocyte diamond: infiltration of mononuclear cells ,open diamond: Inflammation; filled box: kupffer cell, H&E stain (×400).

CONCLUSION

This study is the first time to demonstrate that coumarin fraction of *citrullus colocynthis* extract possess significant antioxidant and anti-uricemic activities in oxonate-treated mice. Further studies on this species may yield fruitful results and isolation of some active constituents may lead to the provision of new natural drugs for treatment of hyperuricemia and gout.

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