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Novel Natural Anti Gout Medication Extract from *Momdica charantia*

Intisar Reehem Alsultanee, Mufeed Jalil Ewadh, Maha Fahdil Mohammed
Babylon University, College Of Medicine , Biochemistry Dept. , Hilla , Iraq
E-mail : mewadh@yahoo.com

Abstract
Medicinal plants were evaluated for their Xanthine Oxidase (XO) inhibitory potential. Their aqueous extracts, prepared from used parts, were tested *in vitro*, at 100 µg/mL concentration, for their inhibition potencies expressed as % inhibition of XO activity. Two of the test plants were found inhibition% activity of xanthine oxidase. Namely *Zingiber officinat* (81.56±3.76) , *Momdica charantia* (96.5±2.17).Total Phenolic of *Momdica charantia* (80.83±0.30) was more than *Zingiber officinat* (62.18±0.65).Fraction of *Momdica charantia* (coumarin) was achieved the highest activity (inhibition activity of xanthine oxidase 97%) based on analysis HPLC. Screening of xanthine oxidase activity by coumarin in terms of kinetic parameter revealed uncompetitive mode of inhibition where K_m 25Mm^{-1},V_{max} 0.38 Mm min^{-1}.Data showed that coumarin (0.5 mg/kg) treatment cause significant reduction in the serum uric acid level of hyperuricemia in normal mice. These finding suggest that *Momdica charantia* extract possess prominent medicinal properties and can be exploited as natural drug to treat diseases associated with free radical formation , oxidative stress and xanthine oxidase activity.
Keywords : Gout , Allpurinol , Xanthine Oxidase , *Momdica charantia*

1. INTRODUCTION

Gout is a common disease with a worldwide distribution. Hyperuricemia, associated with gout, is present in 5-30% of the general population [1]. It seems to be increasing worldwide and is considered an important risk factor in serious disorders like to phaceous gout, gouty nephropathy and nephrolithiasis18-20. Hyperuricemia results from the overproduction or under excretion of uric acid and is greatly influenced by the high dietary intake of foods rich in nucleic acids, such as meats, leguminous seeds and some types of seafood. During the last step of purine metabolism, XO catalyses the oxidation of xanthine and hypoxanthine into uric acid .

Uricosuric drugs which increase the urinary excretion of uric acid, or XO inhibitors which block the terminal step in uric acid biosynthesis, can lower the plasma uric acid concentration, and are generally employed for the treatment of gout [2]. Moreover, XO serves as an important biological source of oxygen derived free radicals that contribute to oxidative damage of living tissues causing various pathological states such as hepatitis, inflammation, ischemia reperfusion carcinogenesis, and aging [3].

Natural products are excellent sources of lead compounds in the search for new medications for some kinds of clinical disorders. The renewed interest in natural therapeutic methods and the use of natural product treatments has led to a steadily growing interest in medicinal plants and the classical methods of plant extract preparations [4].However, systematic exploitation of these natural resources for their human health benefits has not been carried out to a significant degree.Most phytoantioxidants belong either to polyphenols or terpenes and form a family of multiple factors from multiple plants .Polyphenols aresynthesized by plants, participate in their metabolism, and contribute to their defense against environmental stresses[5].

Allopurinol is the only clinically used XO inhibitor in the treatment of gout [6]. However, this drug causes many side effects such as hepatitis, nephropathy, and allergic reactions [7]. Thus, the search for novel XO inhibitors with higher therapeutic activity and fewer side effects are desired not only to treat gout but also to combat various other diseases associated with XO activity.

The main aim of the present study was to screen some plant species growing wild with respect to their XO inhibition activity as potential sources of natural XO inhibitors which may be potentially useful for the treatment of gout or other XO- induced diseases. The extracts of ten different plants were tested as potential inhibitors of XO enzyme.

2. MATERIALS AND METHODS

2.1. Plant Materials

Plant material consists of dried powered leaves of celery (*apium gravelens)*, parsly (*petroselium crispum*) ,rosemary , seed of flax (*linum* usitatissmun) , pumpkin (*cucurbita pepo)* , rhizome of ginger (*zingiber officinale rose)* , turmeric (*curcurma longa) ,cinnamon ,* pulp of bitter gourd (*momodica charantia)* , . These plant were collected from different geographical places of Asia. The plant materials were cleaned , soaked in water and oven dried .The powder materials were stored in a dark place , at room temperature , until extraction.

2.2. Plant Extraction

The plant were 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 48hrs was incubated in room temperature .The extract was filtered using Whatman No.1 filter paper , the solution was evaporated by rotary evaporator in 40 ºC .All extract were kept in vacuum desiccators over anhydrous calcium chloride and were kept in the fridge in 4 ºC to be test.

2.3. 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 Umamaheswari M., *et al* **[8]**. 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 sample 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 $=(A_{\mathbf{control}}-A_{\text{sample}}) / A_{\text{control}} \times 100\%$ **[9]**.

2.4. Determination of total Phenolic contents in extract plant

Total Phenolic content in extract were estimated by Folin-Ciocalteau reagent as method described by Singleton& Rossi (1965) **[10]**. Gallic acid stock solution (1000 µ g/ml) was prepared by dissolving 100mg of gallic acid in 100ml of ethanol. Various dilution of standard gallic acid were prepared by mixing folin reagent with phenol reagent (1:1), and diluted 1:1 in distilled water , before use.

Calibration curve was plotted by mixing 1ml aliquots of 1.o,2.5,5.0,10,25,50 &100 µ g/ml of gallic acid solutions with 5.0 ml of folin-Ciocalteu reagent and 4.0 ml of sodium carbonate solution (75g/l). the absorbance was measured after 30 min at 20^0 C at 765 nm.

One ml of extract (1.0g/100ml) was mixed separately , with the 5.0 ml of Folin reagent diluted and 4.0 ml of sodium carbonate solution as did in construction of calibration curve , and after 1hour, the absorbance was measured after 30 min at 765 nm. for the determination of total Phenolic compound in the extract by using formula:

C=C1*V/m

C=total content of Phenolic compounds in mg/g, in GAE .

C1=the concentration of Gallic acid from the calibration curve in mg/ml.

V=the volume of extract in ml. M=the weight of plant extract in g.

2.5. Phenolic compound Analysis

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-VIS 10A-SPD spectrophotometer. Standards of suspected compound were run similarly for identification and quantification ,concentration of each isolated compound.

2.6. Fractionation of plant extract

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

2.7. The kinetic inhibition to XO

The kinetic conducted only selected fraction of plant , which gave the highest inhibition effect. To determine the mode of inhibition by active compound from the plant , Line weaver-Burk plot analysis was performed .This kinetics study was carried out in the absence and presence of active compounds with varying concentration of xanthine (20 µg/ml , 40 µg/ml, 50 µg/ml ,75 µg/ml, 100 µg/ml) as the substrate.

2.8. Laboratory Animals

Albino Swiss male mice (30) were obtained from National Center for Drug Control and research / Ministry of Health / Baghdad. 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 .

2.8.1. 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 **[12]** .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.

2.9. Experimental design

Mice were randomly divided into four group equal group (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 coumarin .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 5 mg/kg Allopurinol. The group 5, each animal was injected intra- peritoneal the same dose of PO and the same dose of coumarine in the same time. The freshly prepared samples were administered to the corresponding groups for 3 days .

2.10. 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 -80ºC for future laboratory measurements.

2.11. 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 (490 – 530) is proportional to the amount of uric acid in the specimen **[13]** .

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**).**

3. RESULTS AND DISCUSSION

3.1. *In vitro* xanthine oxidase inhibitory (XOI) activity

Xanthine oxidase is the enzyme that catalyzes the metabolism of hypoxanthine to xanthine and then xanthine to uric acid in the presence of molecular oxygen to yield superoxide anion and hydrogen peroxide **[14]** that contribute to oxidative damage of living tissues **[3]**. It has been shown that XO inhibitors may be useful for the treatment of hepatic diseases, gout, which are caused by the generation of uric acid and superoxide anion radical **[15]**.

In this study, the extracts of 9 different plants belonging to different families were investigated as potential XO inhibitors. The selected plants and their XO inhibition assay results are summarized in Table(1)The degree of XO inhibition was evaluated for all extracts at concentration of 100 µg/ml.

The highest XO activity was shown by 70% methanol extract of *Momodica charantia* (bitter) with 96.5±2.17 comparison with positive control (Allopurinol) significant ≤ 0.001, *Zingiber officinat* (ginger) 81.56±3.76 comparison with Allopurinol significant ≤ 0.01, . Leaf of *Cinnamomum cinnamon*, *Curcurma longa* (turmeric), leaves of *Apium gravelen* (celery),leave of *Petro selium* (parsly) , seed of *Cucurbita pepo* (pumkin),seed of *Linum usitatissmum* (flax), leaves of *Rosemer*y have shown no inhibition of xanthine oxidase activity . Plup of *Momodica charantia* (bitter) have demonstrated the best source of raw material for obtaining the XOI compound as each exhibits more than 90% inhibition of XO under extraction solvents. In fact, plup of plant under evaluation have shown considerable activity for XO inhibition ,substantiate the fact that secondary metabolites in the plup contain diverse classes of bioactive Phenolic compounds such as polyphenols, tocopherols and alkaloids **[16]**, which may act as XOI.

Table (1). Comparison of xanthine oxidase inhibitory activity of crude extracts of ten parts of plant using 70% methanol as the extraction solvent .with positive control (Allopurinol)

Extract of plant	Parts of plant	% xanthine oxidase inhibition
1-*Zingiber officinat* (ginger)	rhizome	81.56±3.76**
2-*Curcurma longa* (turmeric)	Whole plant	28.31±7.70***
3-*Cinnamomum cinnamon*	leaf	44.34±3.96 ***
4-*Apium gravelen* (celery)	leaves	37.92±4.18***
5-*Petro selium* (parsly)	leaves	28.63±2.91***
6-*Momodica charantia* (bitter)	plup	96.5±2.17***
7-*Cucurbita pepo* (pumkin)	seed	27.33±4.46***
8-*Linum usitatissmum* (flax)	seed	36.66±2.99 ***
9-*Rosemery*	leaves	40.33±3.51***
10-Allopurinol (positive control)	-	69.33±4.04

Result are expressed as mean ±SD(n=3),**indicates p≤ 0.01, ***indicates p≤0.001

3.2. Comparison of XO inhibitory activity between allopurinol and optimized extract

The XO inhibitory activity of allopurinol and optimized 70% methanol extract of *Momodica charantia* (bitter) at various concentrations was represented in Table 2. Each has demonstrated more than 50% XO inhibition starting from a concentration of 50 µg /ml. The highest residual or percentage difference between allopurinol and the optimized sample was at 50.00 µg/ml with 62.70%, followed by 75 µg/ml with 79.46% and 100 µg/ml with 96.5%. The high significant at various concentration with Allopurinol p≤ 0.001. Overall, these small percentages are encouraging for *Momodica charantia* (bitter) to be utilized for large-scale purposes for the production of anti-gout supplement, an alternative to allopurinol. In folk medicine, *Momodica charantia* (bitter) has been used as an important traditional herbal medicine due to its vast bioactive compounds found including kaempferol,quercetin, 5, 7-dimethoxycoumarin, alkaloids,carpaine and pseudocarpaine. Any of these bioactive compounds may contribute to XO inhibitory activities, they have previously received considerable attention because of their physiological functions such as antioxidant activity **[16]**.

Table (2). XO inhibitory activity of allopurinol and optimized methanol extract of *Momodica charantia* (bitter) , *Zingiber officinat (ginger*), at various concentrations.

Extract of plant	% xanthine oxidase inhibition		
	50 µg/ml	75 µg/ml	100 µg/ml
1-*Zingiber officinat (ginger*)	53.12± 3.03 **	71.34 ± 3.72 ***	81.56±3.76**
2- *Momodica charantia* (bitter)	62.70±3.90***	79.46±0.51***	96.5±2.17***

Result are expressed as mean ±SD (n=3) ,**indicate p≤0.01.***indicate p≤0.001.

3.3. Determination of total Phenolic contents in extract plant

Table 3- showed the contents of total phenolic as mg gallic acid equivalent/100 g dry weight of different studied plants. *Momodica charantia* (bitter)showed the highest content of total phenolic compounds (80.83±0.30mg/100 g sample) followed by *Zingiber officinat (ginger*) (62.18±0.65), was of the lowest content of phenolic compounds.

Table (3). Total phenolic contents of plants under investigation.

Extract of plant	Total Phenolic (mg gallic acid equivalent /100g)
1-*Zingiber officinat* (ginger)	62.18±0.65
2- *Momodica charantia* (bitter)	80.83±0.30

Result are expressed as mean ±SD

Previously *Momodica charantia* (bitter) has been shown to contain different phenolic compounds such as carnosol, rosmarinic acid and carnosic acid in addition of their terpene metabolites, and flavones **[17]**. Carnosol has been reported to possess antioxidant and anti-inflammatory activity **[18] .** Rosmarinic acid and carnosic acid have been shown to have very high antioxidant activity **[19]**.

3.4 Phenolic compound Analysis

Results obtained by HPLC shown that *Momodica charantia* (bitter) extract had Phenolic compounds which were (coumarin , tannin , glycoside , flavonoids) figures (1 and 2) The Phenolic compounds recovered from column C-18 and the concentration of these compounds showed in table (4).These result discovered high differentiation between concentration of these compound , coumarin (156.6) than other compound followed by

tannin (46.42). The result revealed multiple peaks contrasted in retention time (4.517,5.22,9.553.10.795) some peaks converged with peaks of retention time for standard solution (4.5 ,5.22,9.553,10.795) for compounds which were injected in same column.

Table (4). The sequence of Phenolic compounds found on the sample of *Momodica charantia* (bitter) extract using HPLC assay.

Subjects	Retention time (minute)	Area µvolt	Concentration µg/ml
1-Tannin	5.22	4615363	46.42
2-Coumarin	4.517	5105033	156.6
3-Flavonoid	9.553	664367	26.5
4-Glycoside	10.795	1380129	23.32

The result suggest that *Momodica charantia* (bitter) act as a good source of natural due to the high levels of tocopherols , alkaloid ,terpen , coumarin ,strong epidemiological evidence shows that compounds may help to protect the human body against damage by reactive oxygen species **[16]**.

Highly reactive free radicals and oxygen species are present in biological free radicals may oxidize protein , lipid can initiate degenerative disease. Antioxidant compounds scavenge free radicals such as peroxide , hydro peroxide and thus inhibit the oxidative mechanism that lead to degenerative diseases **[20]**.

From the above argument we can suggest that compounds in the *Momodica charantia* (bitter) extract had protective effect against gout and other disease originate from free radical because these compound have free radicals sc avenger activity.

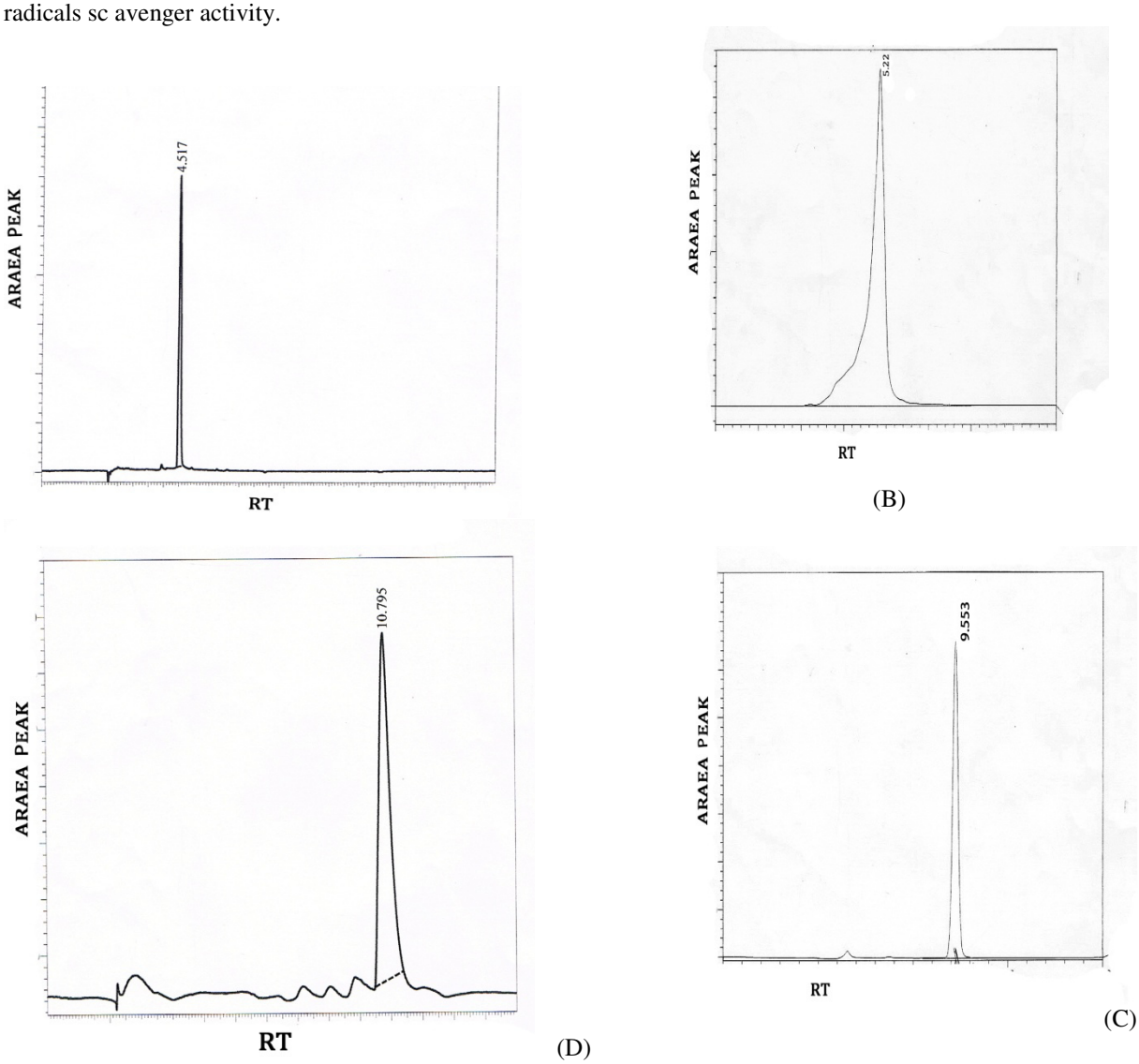

Figure (1). standard of Phenolic compounds

used in the study, (A),standard of coumarin, its retention time (RT) is 4.517.(B), standard Of tannin ,RT is 5.22.(C),standard glycoside , RT is 10.795.(D), standard of flavonoids ,RT is 9.553.and analyzed by HPLC .

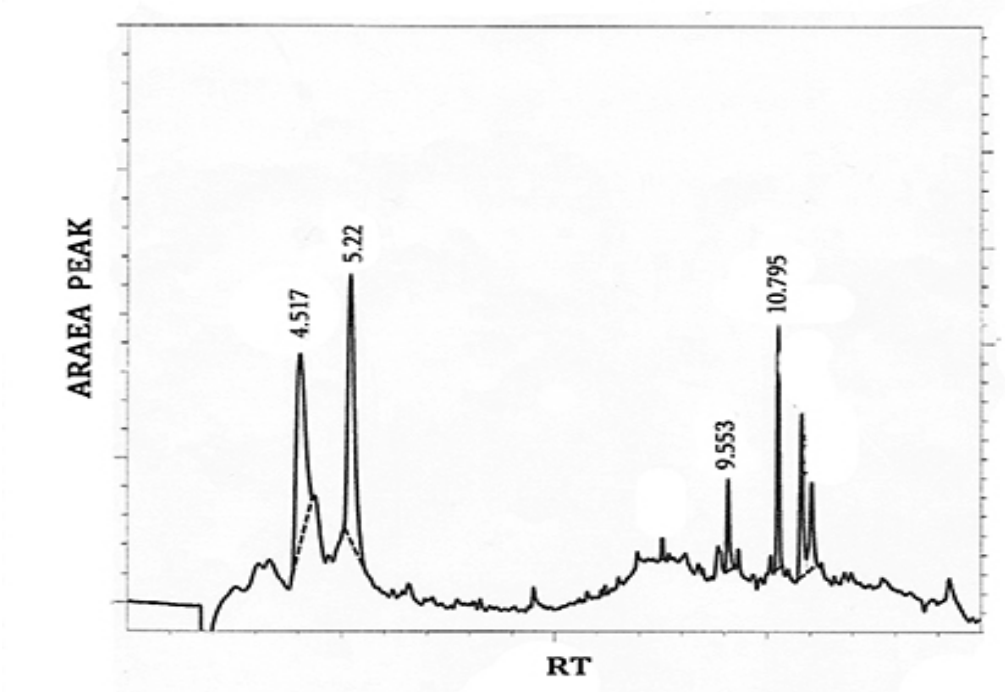

Figure (2). chromatogram of HPLC for sample of *Momodica charantia* (bitter) the RT for coumarin is 4.517 , tannin is 5.22 , flavonoids is 9.553 , glycoside is 10.795.

3.5. Fractionation of plant extract

Fractionation of methanol *Momodica charantia* (bitter) extract was done in mixture of eluents to obtain good separation pattern. Fractionation of 10 mg extract resulted four fractions and gave the fraction (coumarin , 5 mg), (tannin, 3mg),(glycoside , 1.5mg), (flavonoids , 1mg), these fraction were assayed for their inhibition activity against xanthine oxidase. Inhibition activity of fraction (coumarin, tannin, glycoside ,flavonoids) is depicted in figure (3).

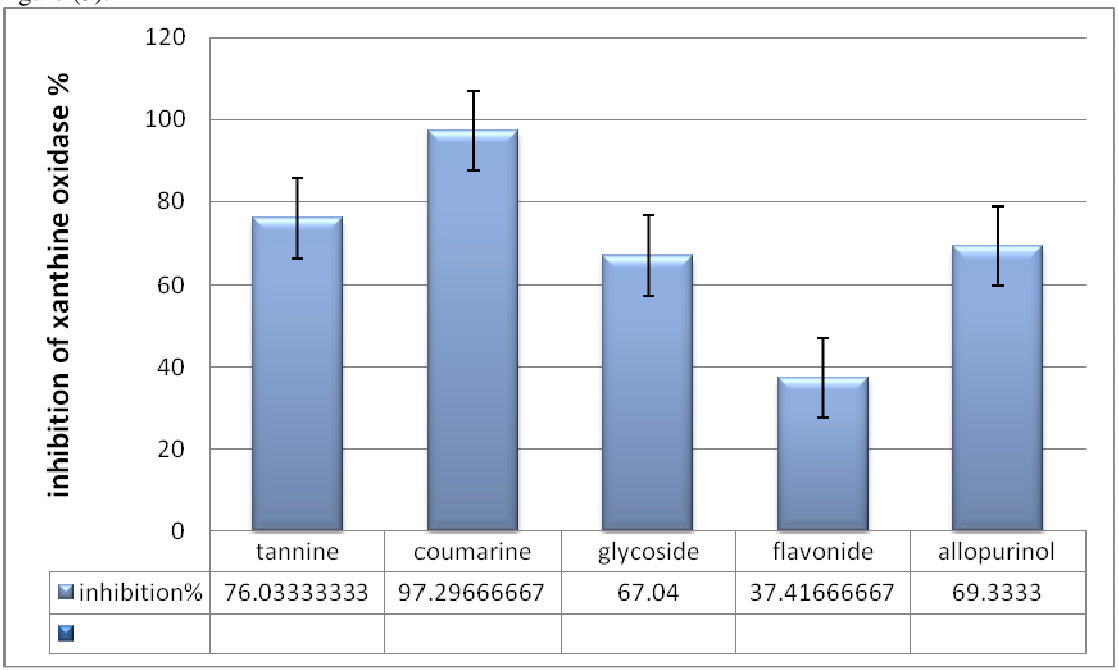

Figure (3). inhibition activity of fractions (coumarin, tannin, glycoside , flavonoids) against xanthine oxidas

The results showed that coumarine had the strongest inhibition activity (97.29 %) against xanthine oxidase , next was tannin and glycoside compared with Allopurinol with inhibition of (69.33%) .The coumarin inhibition activity is suspected to be caused by existence of active compound in coumarin which was potent antigout.

3.6. The kinetic inhibition to xanthine oxidase

Coumarin having the highest inhibition activity against xanthine oxidase was assayed for inhibition kinetic .Figure (4) showed the Line weaver-Burk plot for xanthine oxidase with addition of coumarin.

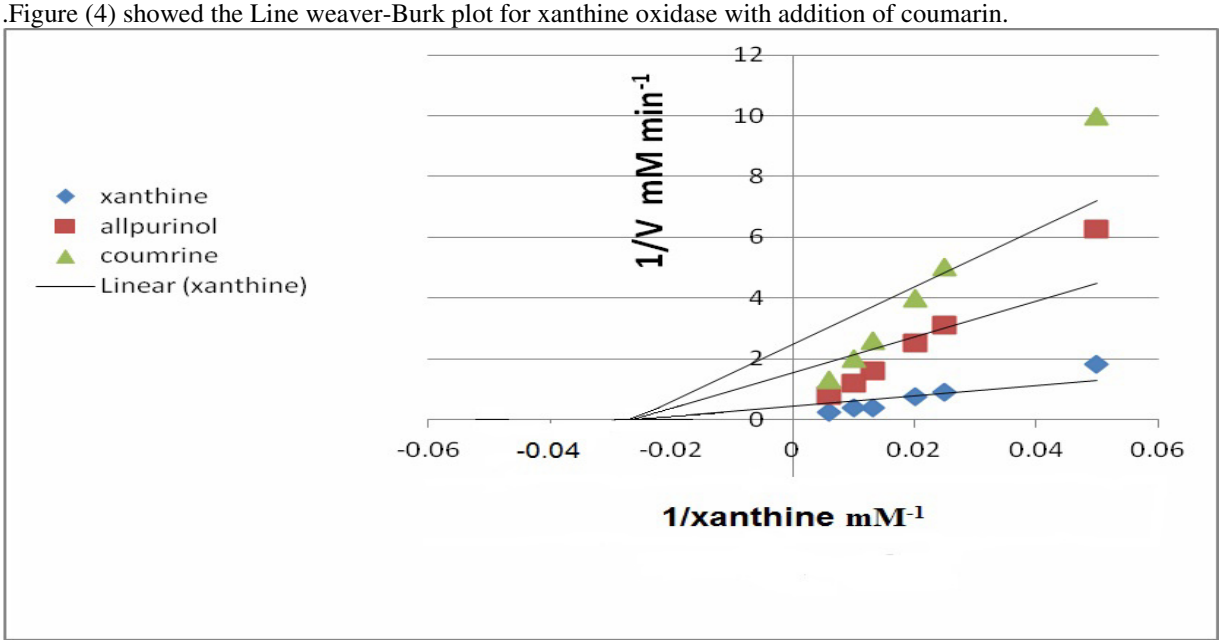

Figure (4). Line weaver-Burk plot of xanthine oxidase activity with coumarin that V_{max} 0.38 mM min $^{-1}$, k_m 25 mM^{-1}

In the kinetic analysis using Line weaver-Burk plot revealed that coumarin displayed high inhibitory activity that V_{max} of coumarin (0.38 mM min $^{-1}$, K_m 25 Mm^{-1}).The pattern of inhibition is a type of un-competitive of inhibition in presence of coumarin were in V_{max} is decreased and K_m appears to be unaltered with respect to xanthine as substrate .

It indicates that binding of coumarin may occur with the free enzyme or the enzyme-substrate complex. The significant inhibition of xanthine oxidase by coumarin may suppress the production of active oxygen species or uric acid in *vivo* under the condition that xanthine oxidase works, the inhibition percentage by coumarin is comparable to that of Allopurinol ,a therapeutic drug used to treat gout.

3.7 Effects of coumarin and allopurinol on serum urate levels in hyperuricemic mice induced by potassium oxonate

As the results presented ,coumarin exhibited a potent XOD inhibitory activity ,the serum uric acid reducing effect by coumarin in oxonate- induced mice by coumarin was further investigated. The serum level of uric acid in mice was induced by uricase inhibitor potassium oxonate (PO).As shown in table 5, initial serum uric acid level in mice was2.47±0.38 mg/dl. After intraperitoneal injection of PO caused a significant increase of serum uric acid level in PO-treated mice ,the level of uric acid was reached at 7.77±0.26mg/dl after injection of PO for 3 hrs.

Table (5) . The hypouricemia effects of coumarin and allopurinol on the serum uric acid levels in hyperuricemia mice

group	Uric acid mg/dl
1-Control	2.47±0.38###
2-Hyperuricemia	7.77±0.26***
3-coumarin	3±0.26*###
4- Hyperuricemia + coumarine	2.93±0.38*###
5-Allopurinol	5.37±0.27 ***###

Result are expressed as mean ±SD (n=6) *indicate p≤0.05,**indicate p≤0.01.***indicate p≤0.001 vs. with control group; # ## indicate p≤0.001 vs. with hyperuricemia group.

After administration of coumarin at a dosage of 0.5mg/kg in hyperuricemia mice, the serum uric acid value was reduced to 3±0.26mg/dl, significant (p ≤0.001) as compared to the hyperuricemia control group .In the same treatment ,allopurinol at a dosage of 10mg/kg, the serum uric acid of mice was reduced to 5.37±0.27 mg/dl .significant (p≤0.001), as compared to hyperuricemia the control group, in group hyperuricemia and coumarin in

same time ,the uric acid level reduced to 2.93±0.38 mg/dl , significant ($p \leq 0.001$) as compared to hyperuricemia .the control group that improvement to kinetic of coumarin. These results suggest that the coumarin were capable of reducing the accumulation of purine metabolites in blood following oxonate induction.

Conclusion

It is concluded that the phytochemical present in this plant extract may also be useful for the treatment of gout and hyperuricemia. The data investigated in this study provides the basis for further investigation on this plant to isolate the active constituents and drug developments against the disease related to oxidative stress, inflammation and gout.

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