Inhibitory Effect of \textit{Ruta graveolens} L. Extract on Guinea Pig Liver and Bovine Milk Xanthine Oxidase

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Abstract

Flavonoids could serve as potent inhibitors of xanthine oxidase (XO). In the present study, the effects of \textit{Ruta graveolens} L. extract and its major isolated flavonoids, quercetin and rutin, on guinea pig liver XO have been investigated. The inhibitory effects of \textit{R. graveolens}, quercetin and its glycoside form, rutin, were assayed spectrophotometrically. \textit{R. graveolens} extract showed moderate inhibition on XO activity. Interestingly, bovine milk and guinea pig liver XO were inhibited significantly at different ranges by either the extract or its flavonoids, whereas allopurinol acted with almost the same potency on both enzymes. Rutin inhibited the enzymes in a competitive manner, while quercetin was found to be a competitive and mixed inhibitor of guinea pig liver and bovine milk XO, respectively. In conclusion, \textit{R. graveolens} extract can act as a good inhibitor of XO. Interestingly, it was shown that the inhibitory effects of flavonoids on XO could be species dependent.

Keywords: Flavonoids; Quercetin; \textit{Ruta graveolens}; Rutin; Xanthine oxidase.

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1. Introduction

Gout and hyperuricemia are metabolic disorders resulted from abnormal production and excretion of uric acid in the body [1]. Xanthine oxidoreductase (XOR) is a cytosolic molybdenum containing enzyme mainly responsible for final reactions of uric acid production from oxidizing oxypurines, hypoxanthine and xanthine [2-5]. The XOR predominantly exists as xanthine dehydrogenase (XD, EC 1.17.1.4) in liver and intestine, but it is occasionally converted to xanthine oxidase (XO, EC 1.17.3.2) either by oxidation of SH-group of cystein residue 535 and 992 [6], reversibly or due to proteolytic reaction with Ca\textsuperscript{2+}-stimulated protease,
irreversibly [7, 8]. Particularly, ischemia-reperfusion significantly predispose the conversion of XD to XO increasing the generation of hydrogen peroxide and superoxide anion which in turn can cause reperfusion injuries [7, 8]. XO also plays an important role in the metabolism of many xenobiotics and drugs such as purines and pyrimidines [9], 6-mercaptopurine and azathioprine [10, 11], thiazides [11], pyrazinamide [12], and acyclovir [13]. Therefore, the inhibition or activation of XO may result in some important therapeutic or toxic effects. Although there are many agents with XO inhibitory effect, apart from allopurinol, none of these inhibitors has implicated for clinical applications. However, allopurinol may cause some serious adverse side effects including, nephropathy, allergic reactions, severe nausea or vomiting and hepatitis [1]. Accordingly, many attempts have been made to find a suitable alternative for allopurinol [14, 15].

Certain aglycons from flavonol subtype have been shown to be potent XO inhibitors by numerous studies [15-26]. However, to date, it has not been addressed the inhibitory effect of certain flavonols on guinea pig liver XO in comparison with bovine milk XO. Guinea pig liver fraction was implied to have similarity to human liver molybdenum hydroxylase [4, 27]. *Ruta graveolens* (Rutaceae) is an herbaceous plant which is rich in flavonoids and has been traditionally used owing to sedative and antihelminitic, hypotensive, antifertility, anti-inflammatory and analgesic actions [28]. The proposed therapeutic effects of *R. graveolens* have been ascribed to some chemical constituents including flavonols [29].

Recently, we have found that *R. graveolens* has a potent inhibitory effects on another molybdenum hydroxylase, aldehyde oxidase (AO, EC 1.2.3.1) [30]. The most important isolated flavonoids in *R. graveolens* were found to be rutin (quercetin-3-O-β-rutinoside) and quercetin (Figure 1). We also indicated that the effects of flavonoids on AO, which has many structural and functional similarities with XO, could be species dependent [31]. To our knowledge, there is no report on the effect of flavonoids and also the *R. graveolens* extract on XO from different sources. In the present comparative study, therefore, the effects of *R. graveolens* extract and its major flavonoids, quercetin and rutin, on XO from bovine milk (as the major source for the preparation of XO) and guinea pig liver (as one of the common laboratory animals used in the experimental studies) have been investigated.

2. Material and methods

2.1. Materials

Allopurinol, xanthine, bovine milk xanthine oxidase (grade I: from buttermilk, 0.5 unit per mg protein) were obtained from Sigma-Aldrich (Poole, Dorset, England). All other chemicals, apart from quercetin and rutin, were purchased from Merck (Darmstadt, Germany).

![Figure 1. Chemical structures of rutin and quercetin.](image-url)
Inhibitory effect of *Ruta graveolens* on xanthine oxidase

### 2.2. Plant material

Leaves of *R. graveolens* were harvested from garden maintained in July 2007 at Tabriz University of Medical Science, Tabriz, Iran. A voucher specimen was deposited with PRTRG001, and a representative of this collection has been retained in the herbarium of the Pharmacognosy Department, School of Pharmacy, Tabriz University of Medical Sciences. Leaves of the plant were dried at room temperature for two weeks and ground using an electric mill.

### 2.3. Extraction preparation and isolation and structure identification of quercetin and rutin

The preparation procedure of total extract with more details was reported in our previous report [30]. In summary, the dried, ground leaves of *R. graveolens* (100 g) were extracted with 70% methanol at room temperature followed by rotatory evaporation in low pressure yielding the dried extract (12.65 g). The dried material was suspended in distilled water, and extracted with petroleum ether, chloroform, ethyl acetate and n-butanol at a maximum temperature of 45°C to obtain the respective fractions. The ethyl acetate fraction was subjected to column chromatography on

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Conc. b</th>
<th>% Inhibition c</th>
<th>IC50 b</th>
<th>Inhibition</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. graveolens</em></td>
<td>100</td>
<td>35.6±4.5c</td>
<td>110±43</td>
<td>59.6±9.3</td>
<td>87.5±2.1</td>
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<tr>
<td>extract</td>
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<td>35.2±11.1</td>
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<tr>
<td></td>
<td>10</td>
<td>15.5±0.1</td>
<td></td>
<td>17.5±10.6</td>
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<tr>
<td>Quercetin</td>
<td>10</td>
<td>95.1±2.6</td>
<td>0.4±0.01</td>
<td>98.2±7.6</td>
<td>0.7±0.1</td>
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<tr>
<td></td>
<td>1</td>
<td>82.4±0.7</td>
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<td>67.3±8.3</td>
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<tr>
<td>Rutin</td>
<td>100</td>
<td>67.2±4.1</td>
<td>39.4±15.8</td>
<td>71.3±2.4</td>
<td>15.8±2.1</td>
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<tr>
<td></td>
<td>10</td>
<td>21.8±8.3</td>
<td></td>
<td>43.8±6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>9.3±1.4</td>
<td></td>
<td>26.5±7.1</td>
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<tr>
<td>Allopurinol</td>
<td>100</td>
<td>94.2±1.4</td>
<td>2.9±0.9</td>
<td>97.1±6.2</td>
<td>2.1±0.8</td>
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<tr>
<td></td>
<td>10</td>
<td>75.3±8.7</td>
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<td>81.5±9.6</td>
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</tr>
<tr>
<td></td>
<td>1</td>
<td>44.1±11.7</td>
<td></td>
<td>46.7±12.3</td>
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</tbody>
</table>

Table 1. IC50 values and inhibitory activity of *R. graveolens* extract, quercetin, rutin and allopurinol on uric acid production progress by guinea pig liver and bovine milk XO a.

Figure 2. Typical Lineweaver-Burk plots for the oxidation of xanthine by bovine milk (a) and guinea pig liver XO (b) in the presence of quercetin.
silica gel using ethyl acetate-methanol-water (70:15:5 v/v/v) as the mobile phase. The flow rate of the organic phase was maintained at 6 drops per min. TLC of column chromatography fractions was carried out on silica gel plates using the mobile phase consisting of ethyl acetate-methanol-water (65:10:15 v/v/v). The main components of the fractions of 55-70 were further purified by preparative TLC on silica gel with a mobile phase of ethyl acetate-methanol (90:10 v/v) which resulted in the isolation of 14.6 mg quercetin. The n-butanol extract was fractioned on a Sep-Pak using a step gradient of 10, 20, 40, 60, 80 and 100% methanol-water mixture (150 ml each) as the eluent. Preparative RP-HPLC (Shim-Pak CLC-C18, 6-150 mm i.d.); gradient elution, 25-70% methanol-water mixture (150 ml each) as the eluent. Preparative RP-HPLC (Shim-Pak CLC-C18, 6-150 mm i.d.; gradient elution, 25-70% methanol in water in 50 min. (20 ml/min.) of the Sep-Pak fraction (60% methanol in water) yielded 82.8 mg rutin. The structures of the both compounds were determined conclusively by UV spectroscopy, 1H- and 13C-NMR analysis [32, 33].

2.4. Preparation of partially purified guinea pig hepatic XO

Partially purified XO were prepared from mature male Dunkin-Hartley guinea pig liver (400-600 g, Tabriz University of Medical Sciences, Tabriz, Iran) according to the method of Johnson et al. [34]. Briefly, the animal was killed by cervical dislocation. Then, the liver was immediately excised and placed in ice-cold isotonic potassium chloride solution (1.15% KCl w/v) containing 0.1 mM EDTA. The liver was homogenized at 3000-4000×g for 1 min. at 4 °C using Potters homogenizer. The homogenate was heated on a steam bath at 55-57 °C, then cooled to 4 °C and centrifuged at 15000×g for 45 min. The supernatant was treated by 50% saturated solution of ammonium sulphate (35.3 g/100 ml) at 4 °C. The resulting suspension was re-centrifuged at 6000×g for 20 min. at 4 °C. The precipitate was dissolved in a minimum volume of 0.1 mM EDTA solution and ultimately they were kept at -70 °C till the time of examinations.

2.5. XO assays

All spectrophotometric determinations were carried out at 37 °C using a Shimadzu UV-visible spectrophotometer UV-2550, which was controlled by the Shimadzu UV Probe personal software package including kinetics software. The instrument was connected to a Shimadzu cell temperature regulatory unit to keep the temperature at 37 °C, constantly. The cuvette used had a path length of 1 cm and the total reaction mixture volume was constant at 3.0 ml. XO activity was assayed by monitoring of uric acid production at 295 nm. Xanthine (50 μM), as specific substrate for XO, was incubated with the enzyme fraction in Sorenson’s phosphate buffer (67 mM, pH 7.0) containing 0.1 mM EDTA and the initial oxidation rates were measured up to 3 min. The reactions were also measured in the presence of different concentrations of R. graveolens extract, quercetin and rutin to assay the inhibitory effects. The results were compared with the inhibitory effects of allopurinol, the potent specific inhibitor of XO [3, 23].

2.6. Determination of kinetic constants

The IC50 values of R. graveolens extract, quercetin and rutin were obtained from the

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Bovine milk XO</th>
<th>Hepatic guinea pig XO</th>
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<tbody>
<tr>
<td>Quercetin</td>
<td>Ki (μM)</td>
<td>KI (μM) Mode</td>
</tr>
<tr>
<td></td>
<td>0.06±0.03b</td>
<td>0.22±0.16 Mixed</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.70±0.10</td>
<td>- Competitive</td>
</tr>
</tbody>
</table>

a The Km and Vmax values for bovine milk and hepatic xanthine oxidase were calculated as follows: 2.9±0.4 μM, 27.4±4.8 μM/min./mg protein and 6.1±2.4 μM, 35.7±3.4 μM/min./mg protein, respectively. b The results are expressed as Mean±S.D, n=3.
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Michaelis-Menten constant (Km) and maximum initial velocity (Vmax) values for the oxidation of xanthine by the enzyme fractions were determined spectrophotometrically from a Lineweaver-Burke double reciprocal plot of 1/v against 1/[xanthine]. The best fit line through the plotted points was calculated using linear regression by the least square method. The reactions were also studied in the presence of quercetin and rutin. The inhibition constants were determined using secondary plot (slopes and intercepts from the initial Lineweaver-Burk plot vs inhibitor concentrations). In the case of mixed inhibition where inhibitor can bind to the free enzyme and to the enzyme-substrate complex, two inhibitor constants were defined: Kᵢ as the dissociation constant of the enzyme-inhibitor complex, and Kᵢ as the dissociation constant of the enzyme-substrate-inhibitor complex. If Kᵢ<Kᵢ, the inhibition was considered as a competitive-noncompetitive type of the inhibition; if Kᵢ>Kᵢ, the inhibition was considered as uncompetitive-noncompetitive type [35].

2.7. Protein determination

Protein concentrations of partially purified enzyme fractions were determined spectrophotometrically using a Pierce BCA Protein assay kit with bovine serum albumin as a protein standard [36].

3. Results

The ¹H-NMR and ¹³C-NMR of the isolated compounds from the extract significantly revealed the chemical shifts of protons and carbons, which is consistent with those reported in the literature for quercetin and rutin [30, 32]. The chemical structures of quercetin and rutin also have been confirmed by analysis of the UV spectra of the compounds with the diagnostic shift reagents [33].

In Table 1, the inhibition of XO by R. graveolens extract and its major flavonoids, quercetin and rutin, in comparison with the inhibitory effect of allopurinol, a potent inhibitor of XO, have been tabulated. The R. graveolens extract inhibited bovine milk XO activity by 37% at 100 mg/ml with an IC₅₀ value of 110 μM. The corresponding values for the inhibition of the guinea pig liver enzyme were 60% and 88 μM, respectively. Bovine milk and guinea pig liver XO were inhibited significantly with 10 μM quercetin (95.1 and 98.2%) and estimated IC₅₀ values were 0.4 and 0.7 μM, respectively. Rutin caused weak inhibition on uric acid production by both enzymes (21.8 and 43.8%) at 10 μM with IC₅₀ values of 39.4 and 15.8 μM, correspondingly. The inhibitory activities of allopurinol against both enzymes were determined at IC₅₀ values of 2.9 and 2.1 μM, respectively.

The inhibitory features induced by quercetin and rutin, on bovine milk and hepatic XO were further investigated by the enzymatic kinetic parameters (Table 2). Quercetin inhibited the bovine milk XO in a mixed manner (competitive-noncompetitive type) with kinetic constants of Kᵢ=0.06±0.03 and Kᵢ=0.22±0.16 μM (Figure 2a). This flavonol inhibited competitively the guinea pig hepatic enzyme at Kᵢ=0.11±0.02 (Figure 2b). Rutin applied its inhibitory effects on the bovine milk and guinea pig liver XO through competitive inhibitory manner with kinetic constants of 0.70±0.10 and 3.01±0.12, respectively (Table 2).

4. Discussion

XO contributes to metabolic clearance of endogenous purines, aldehydes and also some important xenobiotics [3]. Additionally, it has been shown that the activity of this enzyme is commonly over-expressed in ischemia-reperfusion injury and some other related clinical complications such as brain tumor, hepatitis, organ transplantations, birth trauma, and severe intense physical activity [7, 37]. Accordingly, there is a keen interest to find an efficient XO inhibitor as a therapeutic agent particularly from natural sources.
Medicinal plants are one of the major options for finding and developing new remedies [1, 38].

There is a large body of evidence indicating that some flavonoids are potent inhibitors of XO, particularly on bovine milk XO [15-26]. *R. graveolens* is an herbaceous plant, which is rich in flavonoids as its major active reagents [28]. More recently, it has been shown that the extract of this medicinal plant can act as an inhibitor of AO, another molybdenum hydroxylase [30]. With this regard, the effect of *R. graveolens* extract on XO activity was investigated and provided evidences indicated that the extract was able to exhibit moderate inhibitions on bovine milk and guinea pig liver XO activities. The inhibitory effect of quercetin on either type of enzyme was found to be relatively higher than that observed with allopurinol, which is consistent with those results reported by the others [18, 23].

There was also a marked potent inhibitory effect of quercetin on XO compared with that of rutin as it has been pronounced by others [23-25]. It is well documented that the bulky glycosyl group substitution on C-3 of quercetin attenuates significantly the binding affinity of the glycosidic flavonoid with the enzyme [16, 23]. The importance of hydroxyl group at C-3 position in C ring of the flavonoid has also been demonstrated for AO. Likewise their effects on XO, guinea pig liver AO is inhibited more markedly by quercetin than rutin [30, 31]. Similar structural concept has also been reported for the inhibition of AO by steroids. The substitution of hydroxyl group at C-3 of A ring of β-estradiol proposed to reduce the inhibitory effect of steroid on AO activity [39].

Interestingly, quercetin and rutin displayed different degrees of inhibition on bovine milk XO versus guinea pig liver XO. In this case, the aglycone (quercetin) caused a potent inhibition on the former enzyme, whereas rutin exert effective inhibition against guinea pig liver XO rather than bovine milk XO (Table 1). However, this different inhibitory activity was not observed, while allopurinol was employed. Indeed, unlike allopurinol, theses flavonoids impart with different mode of inhibition on bovine milk and guinea pig liver XO activities. The enzymatic kinetic parameters indicated that quercetin inhibited bovine milk XO in a mixed manner, while the guinea pig hepatic enzyme was inhibited competitively by this flavonoid. It is possible that bovine milk XO compared with the guinea pig liver enzyme has an extra site in addition to the active site for binding of quercetin, which results more influential inhibition on bovine milk XO (smaller IC$_{50}$ value) than the guinea pig hepatic enzyme. Rutin exerted its inhibitory effect on the bovine milk and guinea pig liver through competitive inhibitions. Although the provided evidences on XO and AO show species variation (the substrate specificity and inhibitory kinetic properties), the XO was shown to be less species- and tissue-variant than the AO [2-4].

Even though it can be concluded that the observed different inhibitory behaviors of the studied flavonoids may associate with the species variations, the chemical structure of inhibitors may also have considerable impact. Hence, the subject of species variation may be clarified by performing further studies with more flavonoids.

### 5. Conclusion

It was shown that *R. graveolens* with high flavonol-content can serve as a good inhibitor of XO. Whether this natural plant can be administered as an alternative to allopurinol, is subjected for further studies. The data reported in the present study indicate that the inhibitory effects of flavonoids on XO could be species dependent. As these natural flavonoids are found ubiquitously in many vegetables, fruits and plant-derived remedies, their effects on XO have received much
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attention from researchers, and it would be of value to perform more studies to find the best experimental animal model for human XO/XD system with respect to these polyphenols.

Acknowledgements

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of *Biota orientalis* extract and its flavonoid constituents, quercetin and rutin on serum uric acid levels in oxonate-induced mice and xanthine dehydrogenase and xanthine oxidase activities in mouse liver. *J Ethnopharmacol* 2004; 93: 133-40.


