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*Review Article*

# Xanthine Oxidase: Isolation, Assays of Activity, and Inhibition

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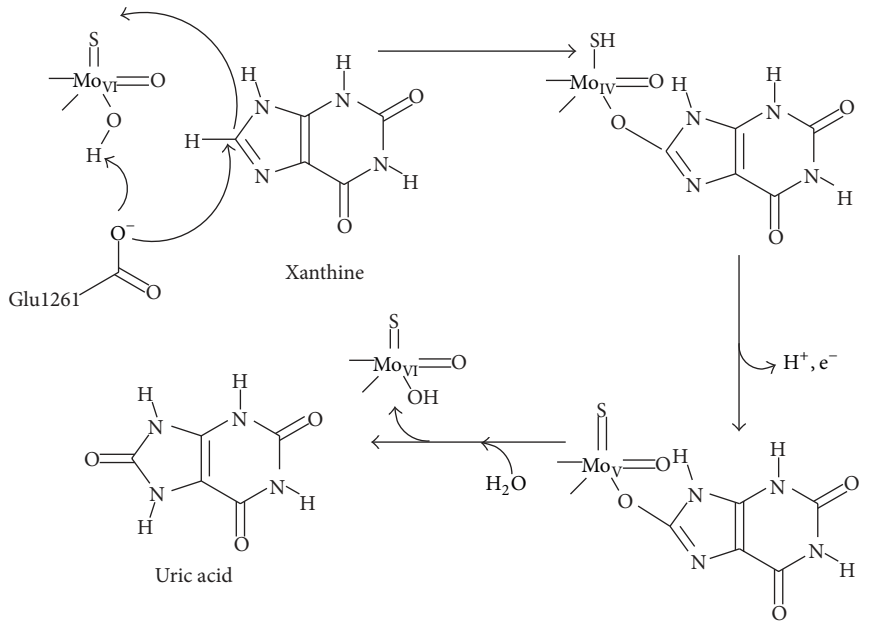
Xanthine oxidase (XO) is an important enzyme catalyzing the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid which is excreted by kidneys. Excessive production and/or inadequate excretion of uric acid results in hyperuricemia. This paper presents a detailed review of methods of isolation, determination of xanthine oxidase activity, and the effect of plant extracts and their constituents on it. Determining the content and activities of XO can be used for diagnostic purposes. Testing inhibition of XO is important for detection of potentially effective compounds or extracts that can be used to treat diseases that are caused by increased activity of XO. *In vitro* bioassays are used to examine test material for XO inhibition, as inhibitors of XO may be potentially useful for the treatment of gout or other XO induced diseases. Several authors reported on the XO inhibitory potential of traditionally used medicinal plants.

## 1. Introduction

Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radical anion, hydroxyl radical, alkylperoxyl radical, nitric oxide, and singlet oxygen are often associated with some physiopathological states in human. Oxidative stress, caused by an imbalance between antioxidant systems and the production of oxidants, including ROS, is considered to contribute to a wide variety of degenerative processes and diseases such as atherosclerosis, Parkinson's disease, Alzheimer's dementia, and reperfusion injury of brain or heart [1, 2] and also can be associated with the pathogenesis of various conditions such as aging, arthritis, cancer, and inflammation [2, 3]. ROS are generated inside the human body as a consequence of the exposure to a multitude of exogenous chemicals in our ambient environment like during UV light irradiation and by X-rays and gamma rays or produced during metal catalyzed reactions [4] and/or a number of endogenous metabolic processes involving redox enzymes and bioenergetics electron transfer [5]. Endogenous factors leading to formation of ROS can be neutrophils and macrophages during inflammation or byproducts of mitochondrial catalyzed electron transport reactions and various other mechanisms [6]. Endogenous sources of ROS include mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation [7], so as other cellular sources of superoxide radicals present such as the enzyme xanthine oxidase, which catalyzes the reaction of hypoxanthine to xanthine and xanthine to uric acid. In both steps, molecular oxygen is reduced, forming the superoxide anion followed by the generation of hydrogen peroxide [8].

Besides these, many studies have now confirmed that exogenic antioxidants, especially supplied by foods, are essential for counteracting oxidative stress. These antioxidants mainly come from plants in the form of phenolic compounds (flavonoids, phenolic acids and alcohols, stilbenes, tocopherols, and tocotrienols) [9]. Many studies have suggested that flavonoids exhibit biological activities, including antiallergenic, antiviral, anti-inflammatory, and vasodilating actions. These pharmacological effects are linked to the antioxidant properties of flavonoids. Protective effects of flavonoids are ascribed to their capacity to suppress ROS formation by inhibiting some enzymes or chelating trace elements involved in free radical production, scavenge radical species and more specially the ROS, and improve regulation antioxidant defense [10–13].

The aim of this study is to give an overview of methods for isolation and determination of XO activity.




Scheme 1: Transformation of xanthine to uric acid by XO.

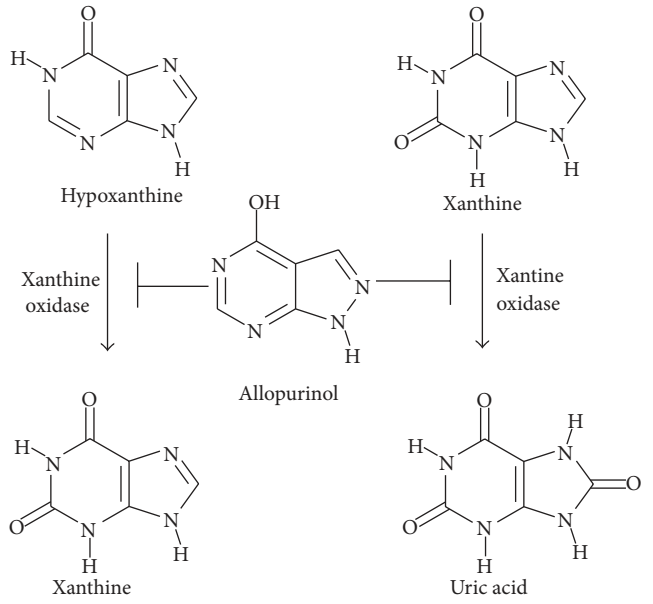



Figure 1: Inhibition of xanthine oxidase by allopurinol to prevent conversion of hypoxanthine to xanthine and/or uric acid.

## 2. Xanthine Oxidase: Mechanism of Action

In 1902, Schardinger [14] showed that milk contains an enzyme capable of oxidizing aldehydes to acids, accompanied by the reduction of methylene blue; this enzyme was then commonly called the "Schardinger enzyme." In 1922, Morgan et al. [15] showed that milk contains an enzyme capable of oxidizing xanthine and hypoxanthine, with the concomitant reduction of $O_2$ to $H_2O_2$, and this enzyme was called XO. Hass and Hill [16] and Hass and Lee [17] reported that milk contains a substance, which they called "itate," capable of oxidizing nitrite to nitrate in the presence of an aldehyde and $O_2$ under other conditions, and that milk could reduce nitrate to nitrite. In 7 1938, Booth [18] presented strong evidence that the Schardinger enzyme was, in fact, XO.

XO is a homodimer with molecule mass of 290 kDa. Xanthine oxidase belongs to the molybdenum-protein family containing one molybdenum, one of the flavin adenine dinucleotides (FAD), and two iron-sulfur (2Fe-2S) centers of the ferredoxin type in each of its two independent subunits. The enzyme contains two separated substrate-binding sites. XO catalysed the oxidation of hypoxanthine to xanthine and

Table 1: Assay procedure of effect of plant extract on xanthine oxidase activity.

| Reference | Tested sample | Assay procedures |
|---|---|---|
| [19] | *Erythrinastricta* Roxb. fractions | Sample fraction (1 mL, 5–100 $\mu$g/mL), phosphate buffer (pH 7.5) (2.9 mL), xanthine oxidase (0.1 mL, 0.1 units/mL in phosphate buffer); preincubation 15 min (25°C); substrate solution (2 mL, 150 $\mu$M xanthine in buffer); incubation 30 min (25°C); HCl (1 mL, 1 M) |
| [20] | Lanostanoids from *Ganoderma tsugae* | Test solution, 70 mM phosphate buffer (pH 7.5); enzyme solution (0.1 units/mL in 70 mM phosphate buffer (pH 7.5)); preincubation 25°C for 15 min; substrate (150 $\mu$M xanthine in the same buffer) |
| [21] | Food extracts and components | Test sample, xanthine (0.8 mL, 150 $\mu$M), hydroxylamine (0.2 mM), and EDTA (0.1 mM), all in sodium phosphate buffer (0.2 M, pH 7.5); XO (0.2 mL, 23.42 mili, 0.2 M phosphate buffer); incubation 30 min (37°C); HCl (0.1 mL, 5 M) |
| [22] | Malaysian medicinal plant | Sodium phosphate buffer (pH 7.5) (300 $\mu$L, 50 mM), 100 $\mu$L of sample solution in distilled water or DMSO, enzyme solution (100 $\mu$L, 0.2 units/mL of xanthine oxidase in phosphate buffer), 100 $\mu$L of distilled water; preincubation 15 min (37°C); xanthine (200 $\mu$L, 0.15 mM); incubation 30 min (37°C); 200 $\mu$L of 0.5 M HCl |
| [23] | Edible plants of the Turkmen Sahra region | Plant extract (0.250 mL in 50 mM potassium phosphate buffer, pH = 7.4), xanthine (0.330 mL, 0.15 mM), potassium phosphate buffer (pH 7.4) (0.385 mL, 50 mM); XO (0.035 mL, in 50 mM potassium phosphate buffer) |
| [24] | Anacardic acid | Sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0) (2.76 mL, 40 mM), xanthine (0.06 mL, 10 mM), sample (0.06 mL in DMSO); xanthine oxidase (0.12 mL, 0.04 units) |
| [25] | Czech medicinal plants | Phosphate buffer (pH 7.5) (400 $\mu$L, 120 mmol/L), xanthine (pH 7.5) (330 $\mu$L, 150 $\mu$mol/L), extract stock solution (250 $\mu$L), enzyme solution (20 $\mu$L 0.5 units/mL in buffer) |
| [26] | *Centaurium erythraea* infusion | Xanthine (44 $\mu$M in 1 $\mu$M NaOH), XO (0.29 units/mL in 0.1 M EDTA), lyophilized infusion (5.2, 10.4, 20.8, 41.7, 83.3, and 166.7 $\mu$g/mL) |
| [27] | Dried *S. anacardium*seeds | 0.1 U/mL XO, 0.2 mL xanthine (0.26 M) in 50 mM phosphate buffer (pH = 7.4) |

subsequently to uric acid [32–34]. During the reoxidation of XO, molecular oxygen acts as electron acceptor, producing superoxide radical and hydrogen peroxide [35]. During these reactions, superoxide anion radicals ($O_2^{\bullet -}$) and $H_2O_2$ are formed [35]. Superoxide anion radicals spontaneously or under the influence of enzyme superoxide dismutase (SOD) transformed into hydrogen peroxide and oxygen. These reactions can be written as follows [36] and on Scheme 1:

$$\begin{gathered}\text{hypoxanthine} + O_2 + H_2O \longrightarrow \text{xanthine} + H_2O_2 \\ \text{xanthine} + 2O_2 + H_2O \longrightarrow \text{uric acid} + 2O_2^{\bullet -} + 2H^+ \\ \text{xanthine} + O_2 + H_2O \longrightarrow \text{uric acid} + H_2O_2 \\ 2O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2\end{gathered} \quad (1)$$

Uric acid is breakdown product of ingested and endogenously synthesized purines. DNA and RNA are degraded into purine nucleotides and bases, which are then metabolized, via the action of xanthine oxidase, to xanthine and uric acid. These later steps are irreversible and generate superoxide anions. Uric acid undergoes no further metabolism in humans and is excreted by the kidneys and intestinal tract [37].

According to this higher concentrations of uric acid may be response to the higher levels of xanthine oxidase activity and to the oxidative stress, which is characteristic for many vascular disease states [38]. The overactivity of XO results in a condition known as gout [39], a common rheumatic disease and an acute inflammatory arthritis [40]. The treatment for hyperuricemia and gout is either increasing the excretion of uric acid or reducing the uric acid production.

Xanthine oxidase inhibitors (XOI) are very useful for this [19]. The inhibition of XO reduces both vascular oxidative stress and circulating levels of uric acid. The inhibition of XO by allopurinol is showed in Figure 1.

Thus, XO inhibitors may be useful for treatment of many other diseases [41, 42]. Among the many known XO inhibitors, allopurinol, oxypurinol, and febuxostat have been used widely for the treatment of hyperuricemia and gout [43]. XO inhibitors can act either at the purine binding site such as allopurinol [44, 45] or at the FAD cofactor site such as benzimidazole [46]. XO inhibitors act by blocking the biosynthesis of uric acid from purine in the body [47] and it is believed that either increasing the excretion of uric acid or reducing the uric acid production helps to reduce the risk of gout [48].

## 3. Isolation and Purification of XO

Isolation of XO, as the widespread enzymes among different species, involves the extraction of the enzyme from a wide range of materials (bacteria, milk, organs of different animals, etc.) and its purification from crude extract. XO is concentrated in the milk fat/lipid globule membrane (MFGM), in which it is the second most abundant protein, after butyrophilin. Therefore, all isolation methods use cream as

Table 2: Plant extract and pure compounds as potent XOI.

| Reference | Plant | Extract or compound | Inhibition effect $IC_{50}$ or % of inhibition |
|---|---|---|---|
| [28] | *Semecarpus anacardium* | Fractionated methanolic extract by hexane, ethyl acetate, and butanol<br>Isolated compound: tetrahydroamentoflavone (THA) | THA<br>$IC_{50}$ 100 nM |
| [29] | *Ajuga iva* L. | Crude 85% methanol extract fractionated by chloroform (CE), ethyl acetate (EAE), and water (AE) | $IC_{50}$ 3.878–5835 $\mu$M/quercetin equivalent (QE) |
| [30] | *Teucrium polium* | Methanol (ME), chloroform (CE), and ethyl acetate (EAE) crude extracts | CE 0.79, ME 10.59, EAE 11.75 $\mu$M/QE |
| [21] | Food extracts | Various extracts and compounds | Hesperetin 35 $\mu$M/QE<br>Theaflavin-3,3′-digallate 49 $\mu$M/QE<br>Cranberry juice, purple grape juice, and black tea $IC_{50}$ 2.4–5.8% of extracts<br>Sage, cinnamon, thyme, and rosemary infusions $IC_{50}$ 12.9–20.4% of infusion |
| [31] | Flavonoids | Genistein, apigenin, quercetin, rutin, and astilbin | *In vitro*: no significant inhibitory effect<br>*In vivo*: quercetin, rutin, and astilbin-potent XOI |
| [20] | *Ganoderma tsugae* Murr. (Polyporaceae) | CE isolated compounds: new lanostanoids: sugaric acids A, B, and C, tsugarioside A, and four known compounds, 3$\beta$-hydroxy-5$\alpha$-lanosta-8,24-dien-21-oic acid, 3-oxo-5$\alpha$-lanosta-8,24-dien-21-oic acid, ergosta-7,22-dien-3$\beta$-ol, and 2$\beta$,3$\alpha$,9$\alpha$-trihydroxy-5$\alpha$-ergosta-7,22-diene<br>ME: two new lanostanoids, sugariosides B and C, and a mixture of the already known compounds 5$\alpha$,8$\alpha$-epidioxyergosta-6,22-dien-3$\beta$-ol and 5$\alpha$,8$\alpha$-epidioxyergosta-6,9(11),22-trien-3$\beta$-ol | Potent XOI |
| [25] | Plant species from Czech Republic | Different crude plant extracts | Methylene chloride, ME of *Populus nigra* and *Betula pendula*, with $IC_{50}$ of 8.3 and 25.9 $\mu$g/mL,<br>80% EE of *Caryophyllus aromaticus* and *Hypericum perforatum*, 50 $\mu$g/mL |
| [19] | *Erythrina stricta* (Papilionaceae) distributed in India, China, Thailand, and Vietnam | Fractions of the hydromethanolic extract of leaves | Chloroform fraction ($IC_{50}$ 21.2 ± 1.6 $\mu$g/mL),<br>pet. ether ($IC_{50}$ 30.2 ± 2.2 $\mu$g/mL),<br>ethyl acetate ($IC_{50}$ 44.9 ± 1.4 $\mu$g/mL),<br>and residual fraction ($IC_{50}$ 100 ± 3.3 $\mu$g/mL) |
| [26] | *Centaurium erythraea* (Gentianaceae) | *C. erythraea* flowering tops infusion<br>Identified compound: several esters of hydroxycinnamic acids, namely, *p*-coumaric, ferulic, and sinapic acids | Noncompetitively inhibiting xanthine oxidase |

the starting material; the cream is washed and churned to yield a crude MFGM preparation, dissociating and reducing agents are used to liberate XO from membrane lipoproteins, and some form of chromatography is used for purification [49].

Since the discovery of XO many authors tried to purify and characterize this enzyme. Schardinger [14] is a partially purified XO and found that reduced activity of the enzyme is not only a consequence of the removal of fat from milk, but also to reduce the concentration of the enzyme on the surface due to the absorption of fat molecules. XO from milk was highly purified by Ball in 1939 [50]. He used continuous steps of centrifugation on milk in order to separate cream layer rich in XO. Ball [50] separated XO from cream. Since then,

XO has been isolated and purified by several authors [51, 52]. Hart and coworkers [53] prepared XO from milk according to the procedure described earlier by Palmer et al. [54]. They separated cream from milk and then added salicylate and EDTA as concentrated solutions to the cream.

The published purification procedures for xanthine oxidase include proteolytic cleavage, calcium chloride treatment, several ammonium sulfate fractionations, dialysis, and several chromatographic steps [55, 56]. Özer et al. [57] isolated XO from fresh bovine milk modifying previous purification procedures to achieve high-yield purification procedure. They added EDTA and toluene in fresh milk, and after the milk was churned and cooled, the $(NH_4)_2SO_4$ was added. The suspension was centrifuged and the precipitate formed was discarded. The supernatant was brought to 50% saturation with solid ammonium sulfate.

Baghiani et al. [29] purified XO from mammalian milk (bovine), in the presence of 10 mM of dithiothreitol, by ammonium sulphate fractionation, followed by affinity chromatography on heparin agarose.

Zhang et al. [58] isolated and purified XO from *Arthrobacter* M3. The *Arthrobacter* M3 culture was transferred into induction medium. The cells were harvested by centrifugation and were resuspended in sodium phosphate.

Table 3: $IC_{50}$ values of flavonoids and allopurinol for inhibition of XO.

| Compounds | XO $IC_{50}$ ($\mu$M) ± SD |
|---|---|
| (±)-Taxifolin | >100 |
| (+)-Catechin | >100 |
| (−)-Epicatechin | >100 |
| (−)-Epigallocatechin | >100 |
| 4′-Hydroxyflavanone | >30 |
| Naringenin | >50 |
| 7-Hydroxyflavanone | 38.0 ± 7.0 |
| Chrysin | 0.84 ± 0.13 |
| Apigenin | 0.70 ± 0.23 |
| Luteolin | 0.55 ± 0.04 |
| Baicalein | 2.79 ± 0.01 |
| 3-Hydroxyflavone | >100 |
| Galangin | 1.80 ± 0.07 |
| Kaempferol | 1.06 ± 0.03 |
| Quercetin | 2.62 ± 0.13 |
| Fisetin | 4.33 ± 0.19 |
| Morin | 10.1 ± 0.70 |
| Myricetin | 2.38 ± 0.13 |
| Allopurinol | 0.24 ± 0.01 |
| Luteolin + epigallocatechin (1 : 1) | 0.76 ± 0.08 |

## 4. XO Activity Assay: Medicinal Importance

Determining the content and activities of XO can be used for diagnostic purposes. The most frequently used method for the determination of XO activity is described by Marcocci et al. [59] and Cos et al. [34]. The measurement is carried out in buffer pH 7.4, which is the most common carbonate or phosphate puffer, at 25 or 37°C. The incubation period is made by various authors from 15 to 30 min. EDTA is often added to complexation of metals present in the test sample. Spectrophotometric determination of XO activity is based on measuring uric acid production from xanthine or hypoxanthine substrate at around 295 nm. The assay mixture always contains xanthine as a substrate and sample. Reaction is initiated by adding the XO. Higher values indicate a pathological condition.

Some studies support the hypothesis that uric acid is connected with elevated vascular events in patients with hypertension, diabetes, and known cardiovascular disease [60–63]. The treatment for hyperuricemia and gout is either increasing the excretion of uric acid or reducing the uric acid production. Xanthine oxidase inhibitors (XOI) are very useful for this [19]. The inhibition of XO reduces both vascular oxidative stress and circulating levels of uric acid. Allopurinol is XOI with high potential. Inhibitory activities of plant extracts and their constituents are compared with the activity of allopurinol as standard. In Table 1 is given the assay procedure of effect of plant extract on xanthine oxidase activity.

Xanthine oxidase inhibitors (XOI) are typically used in the treatment of nephropathy and renal stone diseases linked to hyperuricemia. There has been recent interest in the potential benefit of XOI in the prevention of vascular disease, because of emerging evidence suggesting a role for serum uric acid in the development of cardiovascular disease; the enzyme is an important source of oxidative stress in the vasculature [64]. XOI are agents that directly inhibit the synthesis of uric acid *in vivo*. Certain active constituents present in crude plant extracts like flavonoids and polyphenolic compounds have been reported to possess XOI [65, 66]. These findings have opened the possibility of isolation of new natural compounds, which can be possible inhibitors of XO, and led to the growing interest in the investigation of medicinal plants. The activity of flavonoids as inhibitors of xanthine oxidase *in vitro* has been reported. The absence of a hydroxyl group at C-3 enhances slightly the inhibition effect on XO [34, 67, 68].

In traditional medicine are used many herbs and their extracts in the treatment of various diseases that are the result of increased XO activity. Scientists have studied why some plants and their extracts have an inhibitory effect on the activity of XO (Table 3) [69].

In Table 2 are given plant extract and pure compounds as potent XOI.

The structure-activity relationship of flavonoids as inhibitors of xanthine oxidase and as scavengers of the superoxide radical, produced by the action of the enzyme xanthine oxidase, was investigated. The hydroxyl groups at C-5 and C-7 and the double bond between C-2 and C-3 were essential for a high inhibitory activity on xanthine oxidase. Flavones showed slightly higher inhibitory activity than flavonols [34].

## 5. Conclusions

This review is an overview of methods for the isolation and determination of XO activity *in vivo* and *in vitro* and inhibition by plant extracts and their constituents. For isolation the most used methods are extraction, centrifugation, and chromatographic separation. Plant extracts and their constituents show good inhibitor activity and therefore may have a positive impact on the prevention of disease caused by increased activity of XO. Elevated concentrations of uric acid in the blood stream of human body lead to formation of gout, characterized by hyperuricemia and recurrent attacks of arthritis, so xanthine oxidase (XO) inhibitors may serve as therapeutic agents for hyperuricemia and/or gout. Xanthine oxidase inhibitors are agents that directly inhibit the synthesis of uric acid *in vivo*. Certain active constituents present in crude plant extracts like flavonoids and polyphenolic compounds have been reported to possess XOI. These findings have opened the possibility of isolation of new natural compounds, which can be potent inhibitors of XO, and led to the growing interest in the investigation of medicinal plants.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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