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Inhibitory effects of flavonoids on molybdenum hydroxylases activity

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Molybdenum hydroxylases, aldehyde oxidase and xanthine oxidase, are metalloflavoproteins that catalyze both oxidation and reduction of a broad range of drugs and other xenobiotics indicating the importance of these enzymes in drug oxidation, detoxification and activation. Both enzymes are also involved in some physiological processes and also the metabolism of some endogenous compounds which may indicate their important roles in *in vivo* conditions. Superoxide radical and hydrogen peroxide produced during molybdenum hydroxylases-catalyzed reactions may be relevant in various disease conditions. Therefore, the interference with the function of molybdenum hydroxylases could be of great importance. Flavonoids are a large group of polyphenolic compounds that are able to interfere with xanthine oxidase and aldehyde oxidase function. As flavonoids are consumed in high content in our daily life, their potential to interfere with molybdenum hydroxylases could be a serious concern for consumer safety. However, the subject has not received enough attention and has usually been overshadowed by that of cytochrome P450 as the most important drug metabolizing enzyme system. The present review focuses on the different aspects of flavonoids interaction with molybdenum hydroxylases considering literature published mainly in the last 2 decades. The review also provides insight into some research areas that may offer a great potential for future studies.

Keywords: aldehyde oxidase, flavonoids, interaction, molybdenum hydroxylase, xanthine oxidase

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1. Introduction

Enzymes are one of the most essential elements that are crucial to life on earth. They are required to maintain virtually every tissue and function and also play an important role in the metabolism of xenobiotics and pharmacokinetic variability of drugs. Accordingly, the interaction of compounds with enzyme systems has created an increasing interest in different aspects of human health. Natural compounds, particularly those that serve as the component of human nutrition, have been the focus of considerable interest of scientific community. Although these compounds are usually considered as the nontoxic part of the human diet, they could interfere with biological and physiological processes and cause a significant change in pharmacological effects of some drugs.

Flavonoids are one of the largest groups of naturally occurring low molecular mass polyphenolic compounds distributed in all geographical zones of the world. They are widely distributed in the plant kingdom as secondary metabolites but the presence and distribution of these compounds vary greatly as a function of taxonomic classes and also plant organ. Flavonoids occur almost in all parts of plants including fruit, seeds, stems, leaves, flowers, roots, bark and wood [1-4].

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Article highlights.

- Flavonoids are one of the largest groups of naturally occurring compounds capable of interfering with the activity of numerous groups of enzymes including molybdenum hydroxylases
- Molybdenum hydroxylases, xanthine oxidase and aldehyde oxidase, are involved in the metabolism of a wide range of xenobiotics and endogenous compounds
- As flavonoids are consumed in high content either in the form of dietary food intake or supplements, their potential to interfere with molybdenum hydroxylases could be a serious concern for consumer safety
- Dietary intake, metabolism and the different forms of a flavonoid that exist in the body play important roles in biological effects of a flavonoid. Therefore, in studying the interaction of flavonoids with any enzyme system including molybdenum hydroxylases, it is very important to consider the pharmacokinetics of flavonoids. This also makes it difficult to extrapolate the results obtained from *in vitro* studies to *in vivo*
- Aldehyde oxidase and xanthine oxidase are homodimeric proteins with many similar physicochemical properties, such as cofactor composition, molecular mass, absorption spectrum and subunit structure
- Aldehyde oxidase- and xanthine oxidase-catalyzed oxidations occur at the molybdenum center transferring one oxygen atom derived from water to the substrate and two electrons into the molecular oxygen as an electron acceptor. Accordingly, both enzymes may serve as an important biological source of some reactive oxygen species involved in many pathological processes
- Aldehyde oxidase and xanthine oxidase can oxidize a wide range of aldehydes and N-heterocyclic compounds with physiological, pharmacological and toxicological relevance
- In spite of this relatively broad spectrum of xanthine oxidase and aldehyde oxidase activities, the interaction of flavonoids with xanthine oxidase in hyperuricemia and gout has been almost the only *in vivo* studies to be considered in the literature. Even so, the results published are controversial
- Compared with xanthine oxidase, there are extremely limited studies concerning the effects of flavonoids on aldehyde oxidase. However, due to extreme similarity of these two molybdenum containing enzymes, it is more likely that those flavonoid-rich plants or pure flavonoids that have been a subject for falvonoid–xanthine oxidase interaction studies may also serve as a good candidate for aldehyde oxidase
- According to some results, the interaction of flavonoids with aldehyde oxidase could be substrate-dependent and the aldehyde oxidase-catalyzed oxidation of aldehydes could be inhibited more potently than N-heterocycle oxidation
- Flavonoids with planar structure are relatively more potent inhibitors of xanthine oxidase and aldehyde oxidase activity than the corresponding flavonoids with non- planar structure. Having a planar structure is crucial for xanthine oxidase, but not aldehyde oxidase, inhibition
- The number and in particular the location of hydroxyl groups on the flavonoid structure have a prominent effect on different inhibitory properties of flavonoids. The largest contribution results from the presence of a hydroxyl group at 5 and/or 7 locations.
- The addition of extra hydroxyl groups at C-3′ and C-4′ positions of B ring of the flavone backbone may slightly increase the inhibitory effect of the flavonoid.
- Existence of a hydroxyl group at 3-position of the flavonoid structure is not essential for the inhibitory activity of the compound against molybdenum hydroxylases; however, substitution of this hydroxyl group either in flavonol or in flavanone backbone leads to a significant reduction in the inhibitory capacity of the flavonoid on both enzymes
- Although aldehyde oxidase and xanthine oxidase have many physicochemical properties in common, based on the limited studies available, the existence of some differences in the interactions of flavonoids and elucidation of these differences can be a challenging subject for the future structure–activity studies
- Taking into account the limited number of studies concerning the flavonoid effects on physiological and pathophysiological processes in which molybdenum hydroxylases play an important role, this research area offers a great potential for future studies
- Although the number of drugs metabolized by molybdenum hydroxylases is few compared to cytochrome P450 family, it seems that the coming years would witness more drugs cleared by these two enzymes, in particular aldehyde oxidase
- In spite of potent inhibitory effects of a large number of flavonoids on molybdenum hydroxylases activity, the interaction of flavonoids with the metabolism of those drugs metabolized by aldehyde oxidase and xanthine oxidase has not received enough attention
- Flavonoids are almost an inevitable part of our dietary program and are expected to be consumed in high content in our daily life; therefore, occurrence of some adverse effects following uncontrolled intake of flavonoids in combination with those drugs that are metabolized by molybdenum hydroxylases could be a serious concern for consumer safety. This is a broad area for scientific studies that has not been covered so far

This box summarises key points contained in the article.

Almost all flavonoids are pigments which are responsible for the wide range of yellow, orange, red, blue and violet color of flowers, fruits, and sometimes leaves, and their colors are undoubtedly associated with some of their important biological functions [1]. This large group of natural compounds possesses a wide range of biochemical and pharmacological properties and a variety of potential beneficial properties of flavonoids such as anti-inflammatory, antibiotic, anticancer and cardiovascular activities have been covered recently in some excellent reviews [3,5].

They also interfere with the activities of many enzymes and this property of the flavonoids may lead to a major problem to the progress and treatment of human diseases. The focus of the present paper is on the effects of flavonoids on two molybdenum containing enzymes, xanthine oxidase and aldehyde oxidase, which are involved in the

metabolism of a wide range of xenobiotics and endogenous compounds [6-10].

2. Flavonoids

2.1 Chemistry of flavonoids

The flavonoid family has numerous members which structurally have two aromatic rings (A and B) linked by a three-carbon chain that is usually characterized by an Ar-C3-Ar carbon skeleton. The three-carbon chain may be open and forms 1,3-diphenyl propane derivatives or takes part in a five- or six-membered heterocycle ring structure (C- ring).

The compounds belonging to the 1,3-diphenyl propane class are regarded as chalconoids and tricyclic derivatives possessing a five-membred heterocyclic ring are classified as auronoids. Finally, the members of Ar-C3-Ar group possessing a six-membered heterocyclic ring are derivatives of phenylbenzopyrans and depending on the position of the linkage of the phenyl (B) ring to the benzopyran moiety, may be subdivided into three distinct classes: 2-phenylbenzo-γ-pyrones (2-phenylchromones) or the flavonoids, 3-phenylbenzo-γ-pyrones (3-phenylchromones) or the isoflavonoids, and 4-phenylbenzo-α-pyrones (4-phenylcoumarins) or the neoflavonoids. Based on the oxidation pattern of the heterocyclic C-ring, these classes may be divided into different subclasses (**Figure 1**) [11]. Furthermore, the various hydroxylation, methoxylation, glycosylation, acylation, prenylation and rarely sulfatation patterns of each of these classes cause much more broad spectrum of structural diversity of flavonoids spread in nature [2,11]. It should be noticed that in some old literature, chalcones, aurones, isoflavonoids, neoflavonoids along with xanthones have been classified as non-true flavonoids.

2-Phenylchromans are the most common class of the natural polyphenols and consist of 11 main subgroups (**Figure 1**).

2.2 Physiological and pharmacological functions of flavonoids

Flavonoids play important roles in plant life as a protecting agent against bacterial, viral and fungal infections and their contribution in UVB protection, pollination and plant–animal interactions is already well established [2]. They also possess several biological activities such as anti-spasmodic, hepatoprotective, anxiolytic, anti-inflammatory, estrogenic, cytotoxic and antitumor, antiallergic, anti-ischemic, immunomodulatory, antibacterial, antifungal and antiviral activities in animal models [12-16]. Flavonoids are most commonly known as efficient antioxidants and the scavenging of oxygen free radicals is considered as one of the most important properties of these natural occurring compounds [3,14,17].

Flavonoids are able to alter the activity of numerous groups of enzymes including hydrolases, oxidoreductases, DNA synthetases, RNA polymerases, phosphatases, protein phosphokinases, oxygenase and amino-acid oxidases [3,18,19]; however, this list is not still complete and there are additional examples of enzyme inhibitions by these substances.

It is thought that some parts of the biological activities of flavonoids are related to their ability to interfere with the activity of some enzyme systems. As mentioned, oxidoreductases are among those enzymes that could be affected by flavonoids. Most biological electron transfer processes require coenzymes of the nucleotide type, although the catalytic function is located in an aromatic moiety. Flavonoids structurally resemble both nucleotides and the aromatic oxidation/reduction catalyst [3]. Therefore, due to this resemblance, it is likely that the competition of flavonoids with the nucleotide for its binding site on the enzyme could account for the inhibitory effects of these important, naturally occurring compounds on oxidoreductases.

2.3 Dietary intake and absorption of flavonoids

The dietary intake and metabolism of flavonoids play an important role in their biological activities. Although the bioactive potential of flavonoids has been recognized for long, little is known about their bioavailability, metabolism, excretion and health effects, and the results published are contradictory [4].

The amounts of daily dietary intake of flavonoids are very variable and it has been roughly estimated to vary from 23 to 1000 mg/day [20-23]. The daily intake of flavonoids can also be affected by the food processing procedure, life style and food habits that may be variable in different communities and different people [24,25]. As a result, it will be very difficult to judge the beneficial health effects of flavonoids based on only their dietary intake.

The extent of absorption of flavonoids is still an unsolved problem. For a long time, absorption of flavonoids from dietary sources was considered to be negligible. However, recent reports suggest that humans absorb appreciable amounts of flavonoids in the gut and could be detectable in plasma and urine of humans [26,27]. Flavonoids present in foods exist predominantly in a glycosylated form rather than in their aglycone form and the latter form is considered as the preferred absorbable form of flavonoids. Accordingly, prior hydrolyzation of the glycosides would be necessary for the absorption of flavonoids. The hydrolysis predominantly occurs in the large intestine as a result of microbial metabolism [28-30], although some flavonoid glycosides could be hydrolyzed in the small intestine or buccal cavity [31]. After hydrolyzation, the aglycones are rapidly reconjugated as glucoronide/sulfate or methylated form by the enzymes including bacterial source located in the small intestine and colon which alter their lipophilicity and are absorbed passively through the epithelium [32,33]. The biological consequences and importance of biotransformation of dietary polyphenols by gastrointestinal micro-flora is complex, which brings about great difficulty in making conclusions [34].

Therefore, flavonoids could exist in several forms and the contribution of each form in the biological effects of flavonoids is not clear and more studies are required to clarify the issue. It would also be difficult to draw definite conclusions

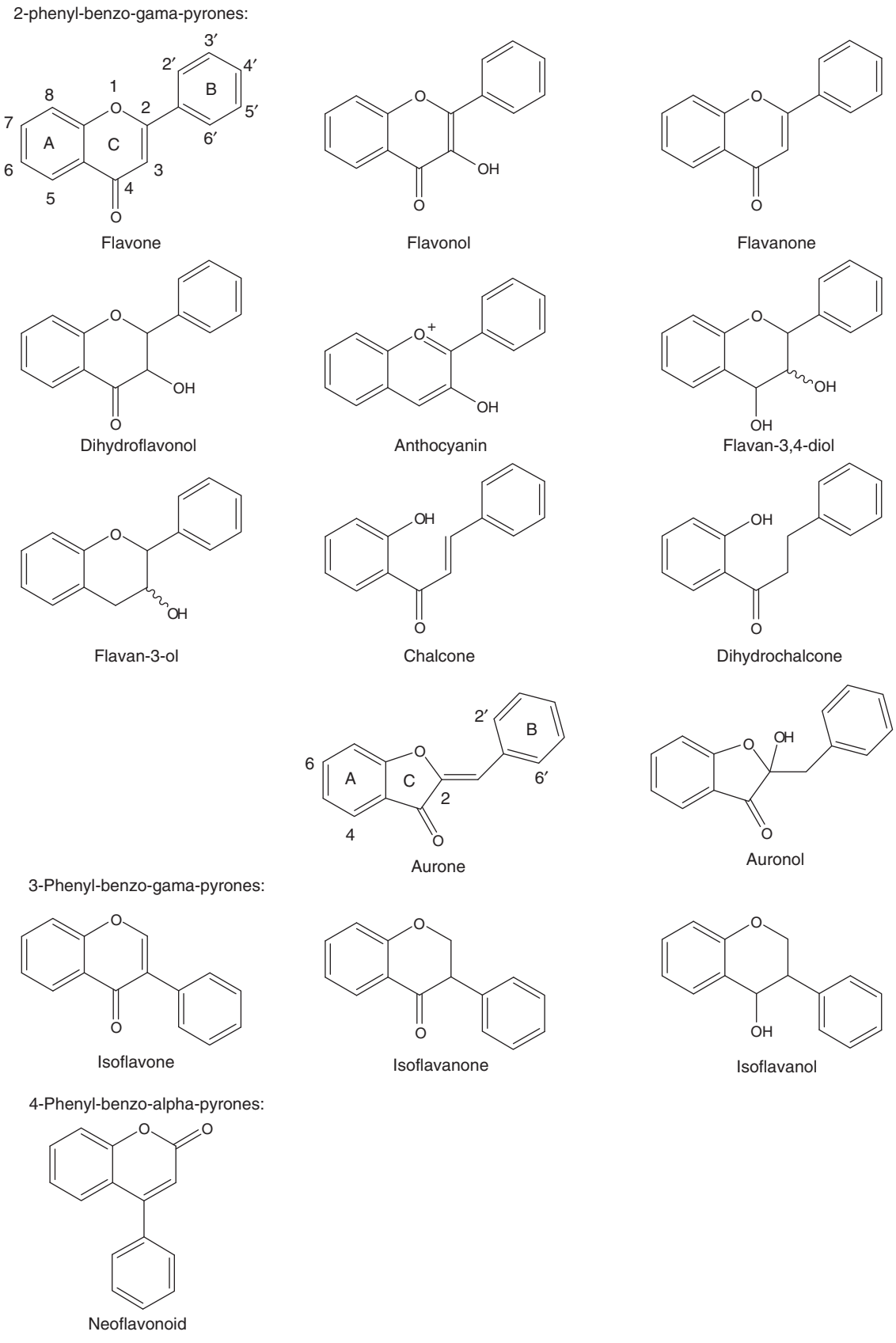

Figure 1. Chemical structures of different classes of flavonoids.

about the interaction of dietary flavonoids with metabolic pathway of drugs.

On the other hand, nowadays, there is an increase in flavonoid intake in the form of dietary supplements and plant extracts due to the presumed association of flavonoids with beneficial effects on health issues. It is, thus, likely that the amount of flavonoid intake exceeds their ingestion with the normal diet by several orders of magnitude [18]. Therefore, the occurrence of some significant drug–flavonoid interactions in those people who take such supplements and plant extracts with conventional drugs at the same time cannot be ruled out. Recently, the interaction of flavonoids with xenobiotic metabolism and some important enzyme systems have been covered in some excellent reviews [5,18].

3. Molybdenum hydroxylases

3.1 General features

Molybdenum hydroxylases are one of the important members of oxidoreductases that comprises of aldehyde oxidase (EC 1.2.3.1: aldehyde: oxygen oxidoreductase), oxidase (EC 1.17.3.2: xanthine: oxygen oxidoreductase)/xanthine dehydrogenase (EC 1.17.1.4: xanthine: NAD^+ oxidoreductase) and sulfite oxidase (EC 1.8.3.1: sulfite: oxygen oxidoreductase). As aldehyde oxidase and xanthine oxidase are the two major members of molybdenum hydroxylases that participate in the biotransformation of xenobiotics, sulfite oxidase is not described here, and the term 'molybdenum hydroxylases' refers to aldehyde oxidase and xanthine oxidase.

Xanthine oxidase and xanthine dehydrogenase are inter-convertible and collectively referred to as xanthine oxidoreductase (EC 1.2.3.2). All these enzymes are referred as molybdenum hydroxylases due to the presence of a unique molybdopterin prosthetic group in their structure [6,35,36]. Here, only aldehyde oxidase and xanthine oxidase are considered and the term 'molybdenum hydroxylases' is used for these two enzymes throughout the manuscript.

Aldehyde oxidase and xanthine oxidase are widely distributed throughout the animal kingdom with no correlation between the presence of one oxidase and the presence or absence of another one [6,37]. Of all the mammalian tissues studied, liver has been found to contain the highest aldehyde oxidase levels, whereas in most species, xanthine oxidase activity is greater in mammary gland and small intestine. Both enzymes are mainly found in the cytosol fraction of tissues [6,38].

3.2 General structural properties

Aldehyde oxidase and xanthine oxidase have many physicochemical properties, such as cofactor composition, molecular mass, absorption spectrum and subunit structure, in common. Both enzymes are large homodimeric proteins with a molecular mass of ~ 300,000 Da, composed of two identical subunits. Each subunit contains one atom of molybdenum, one molecule of enzyme-bound flavin adenine dinucleotide (FAD) and two iron–sulfur (2Fe–2S) centers [38,39]. All these centers are present in different domains within a single polypeptide chain [6,40]. The iron–sulfur center is connected to the FAD binding domain by a linker peptide in such a way that the flavin ring is positioned in close proximity. There is another linker peptide that connects the FAD domain with the C-terminal portion of the protein. This portion of the enzyme contains the molybdenum center and the substrate binding pocket [41,42]. The molybdenum atom is vital to the oxidative capacity of the molybdenum hydroxylases and its removal leads to the inactivation of the enzyme [43].

Apart from nitrogenase in bacteria in which molybdenum is bound to protein via an 'iron-molybdenum cofactor', these molybdenum-containing enzymes have the metal as a part of a 'molybdenum cofactor' in which molybdenum is bound to a unique organic moiety termed molybdopterin [35,36]. The molybdopterin cofactor is tightly bound to the protein through non-covalent interactions and it can be extracted from the enzyme only under denaturing conditions and disruption of molybdenum ligand field [44].

The active site of molybdenum hydroxylases, in the Mo(VI) state, possesses a molybdenum atom in a square pyramidal geometry which is coordinated by two sulfur atoms from one pyranopterin ligand, one terminal oxygen ligand (Mo=O), one terminal sulfido ligand (Mo=S) and one hydroxyl/water moiety [45].

The three prosthetic groups of molybdenum hydroxylases can participate in redox reactions and exist in the several paramagnetic states during the catalytic cycle of enzyme including Mo(V), $FADH^{\bullet}$ and a reduced form of two iron–sulfur centers [35].

3.3 General mechanism of action

Aldehyde oxidase and xanthine oxidase can catalyze the oxidation and reduction reactions [6-9], although the former reactions are much more common than the latter ones *in vivo*. Compared with other drug metabolizing enzymes, such as cytochrome P450, the contribution of aldehyde oxidase and xanthine oxidase, particularly the former enzyme, to drug metabolism has not received enough attention and relatively little is known about these two enzymes.

The reaction catalyzed by aldehyde oxidase and xanthine oxidase is of interest in that unlike other biological hydroxylation systems, the ultimate source of the oxygen atom inserted into substrates is water, not molecular oxygen, and the reaction generates rather than consumes reducing equivalents in the course of substrate hydroxylation [38,45].

The reaction catalyzed by molybdenum hydroxylases can be represented by the general equation of:

$$RH + H_2O \rightarrow ROH + 2e^- + 2H^+$$

where RH and ROH are the substrate and the hydroxylated metabolite, respectively. The equation implies that the oxygen

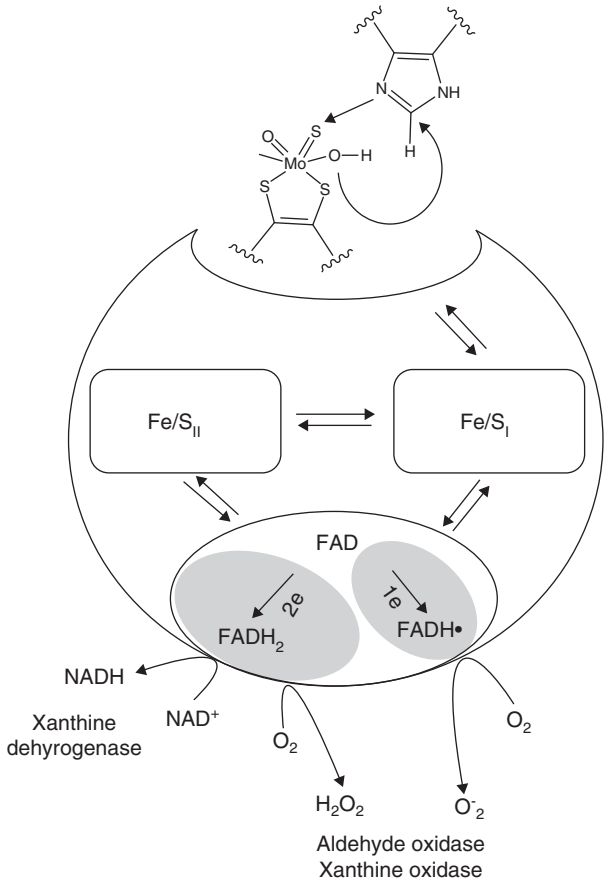

Figure 2. Reaction mechanism and electron transport in molybdenum hydroxylase-catalyzed oxidation reactions at molybdenum and FAD sites [6,35,38,45].

FAD: Flavin adenine dinucleotide.

atom transferred to the substrate is ultimately derived from water and two electrons are released during the reaction requiring an electron acceptor. The reaction occurs at the molybdenum center of the enzyme and during this reaction Mo(VI) is reduced to Mo(IV) (**Figure 2**) [6,35,38,45].

Aldehyde oxidase and xanthine oxidase use molecular oxygen as an electron acceptor. When molecular oxygen acts as an electron acceptor, it undergoes a two-electron reduction to produce hydrogen peroxide; however, a portion undergoes one-electron reduction to produce superoxide anion [35,42,46]. These reactions occur at FAD center and lead to generation of reactive oxygen species (ROS). Hydrogen peroxide is produced via reaction of fully reduced flavin ($FADH_2$) with molecular oxygen, whereas superoxide anion results from the reaction of flavin semiquinone ($FADH^{\bullet}$) with molecular oxygen:

$$FADH_2 + O_2 \rightarrow FAD + H_2O_2$$
$$FADH^{\bullet} + O_2 \rightarrow FAD + O_2^{-}$$

However, the dehydrogenase form of xanthine oxidase, xanthine dehydrogenase, utilizes NAD^+ as the electron acceptor [35,46]. In fact, xanthine dehydrogenase and xanthine oxidase are two different forms of the same enzyme and can be converted to each other in either *in vitro* or *in vivo* conditions [47].

In contrast to xanthine dehydrogenase/oxidase, aldehyde oxidase seems to be a permanent oxidase, with no activity towards NAD^+ [48], and attempts to convert non-proteolyzed aldehyde oxidase to a dehydrogenase form with dithiothreitol have been unsuccessful [49].

3.4 Substrates and inhibitors of molybdenum hydroxylases

Aldehyde oxidase and xanthine oxidase can oxidize a wide range of aldehydes and N-heterocyclic compounds with physiological, pharmacological and toxicological relevance. Although both enzymes have similar structural properties and some overlap in substrate recognition, there are major differences between two enzymes in substrate and inhibitor specificities as well as product regiospecificities [6,10,38]. Aldehyde oxidase is capable of oxidizing a more diverse range of compounds than xanthine oxidase [6,38]. This broad range of substrate specificity indicates that this enzyme has a relatively large active/binding site with a marked flexibility compared to xanthine oxidase. This could be one reason why different substrates such as phenanthridine [50,51], N-methylnicotinamide [52,53], phthalazine [6,54], benzaldehyde [55,56] and vanillin [57,58] have been used to assay aldehyde oxidase activity whereas xanthine oxidase activity has been routinely monitored with xanthine.

In addition, species variation in the levels of aldehyde oxidase may be more pronounced than that of xanthine oxidase [6]. This marked variation in substrate specificity of aldehyde oxidase would make it difficult to extrapolate the results obtained from one animal to another. For instance, carbazeran is a good substrate for guinea-pig, baboon and human liver aldehyde oxidase, whereas it does not serve as a substrate for rabbit enzyme [6]. Conversely, methotrexate is a more efficient substrate for rabbit liver aldehyde oxidase than that of other species [59].

Furthermore, the site of substrate oxidation catalyzed by aldehyde oxidase may be species-dependent. N^1-methylnicotinamide is oxidized to two metabolites, a 2-pyridone (N^1-methyl-2-pyridone-5-carboxamide) and a 4-pyridone (N^1-methyl-4-pyridone-3-carboxamide) by liver aldehyde oxidase from various species; however, the ratio of the 4-pyridone to the 2-pyridone metabolite differs from one species to another [60].

4. Interaction of flavonoids with molybdenum hydroxylases

Xanthine oxidase is the major enzyme in the oxidation of purines to uric acid [61]. High concentration of urate may

lead to some serious problems such as gout. As mentioned before, during the oxidative reactions catalyzed by molybdenum hydroxylases, two electrons are generated; therefore, both xanthine oxidase and aldehyde oxidase could serve as important biological sources of ROS which are involved in many pathological processes such as inflammation, atherosclerosis, cancer and aging [62-64]. The involvement of xanthine oxidase in the urate production and the generation of ROS have led to great efforts to inhibit the enzyme activity to conquer these problems. There is a large body of evidence indicating that flavonoids and other polyphenols such as tannins could be potent inhibitors of xanthine oxidase [58,65-71]. However, it does not mean that the presence of high amount of such polyphenols could be indicative of their high molybdenum hydroxylases inhibitory activity *in vivo*. It has been shown that quercetin pretreatment does not reduce significantly hepatic xanthine oxidase activity in spite of its significant increase in liver after intragastric administration [72]. It has also been indicated that luteolin which is a potent inhibitor of xanthine oxidase [66,68] does not produce any observable hypouricemic effects after acute oral treatment in potassium oxonate-treated rats [73]. In addition, according to some studies, the isolated flavonoids or flavonoid-rich foods have indicated hypouricemic action which could be at least partly due to their inhibitory effects on xanthine oxidase, but a part of other studies had negative results [67,74]. In a study carried out by Kong *et al.* [67] on 122 traditional Chinese medicinal plants, it was shown that despite the desired effects of these plants in alleviation of the signs of illness of gout and hyperuricemic condition, most surveyed plants had no effects on xanthine oxidase activity suggesting alternative mechanism(s) for their action.

The ability of flavonoids to hinder the generation of ROS through xanthine oxidase inhibition should also be carefully considered. Flavonoids could exert their antioxidant properties through scavenging the already formed ROS and/or inhibiting ROS production [65]. Xanthine oxidase is one of the endogenous sources of ROS. According to some authors [65], flavonoids may reduce ROS generation at an earlier stage by inhibiting of xanthine oxidase activity; however, the antioxidant properties of flavonoids may not be necessarily parallel to their inhibitory effects on xanthine oxidase [75] and the attribution of some beneficial effects of flavonoids to their inhibitory effects on free radicals formation by xanthine oxidase could not be straightforward. It has been shown that some flavonoids with a high ability to scavenge free radicals devote some xanthine oxidase activity; meanwhile, some flavonoids are potent inhibitors of xanthine oxidase activity with no free radical scavenging property [75].

In spite of these findings, the interaction of flavonoids either in some flavonoid-rich foods and plants or as pure compounds with xanthine oxidase has been the subject of considerable scientific interest.

4.1 Effects of flavonoids on xanthine oxidase activity: *in vitro* studies

Although xanthine oxidase is involved in several physiological and pathophysiological processes, attention has been paid to the interaction of flavonoids with xanthine oxidase in hyperuricemia and gout.

Sinofranchetia chinensis D.C. (Lardizabalaceae) is used in China as a traditional herb in the treatment of dysuria and painful joints of the limbs [76]. Kong *et al.* have been able to isolate liquiritigenin and isoliquiritigenin from the methanolic extract of the stem of this plant [76]. They showed that these two flavonoids can inhibit xanthine oxidase activity in a dose-dependent manner with IC_{50} values equal to 49.3 μM (for liquiritigenin) and 55.8 μM (for isoliquiritigenin). Both compounds inhibited the enzyme in a mixed type manner.

Hexachlamys edulis (Myrtaceae) is a Paraguayan herbal remedy which has considerable amount of flavan-3-ols and flavonol derivatives in its leaves. Theoduloz *et al.* could show xanthine oxidase inhibitor activity of the plant *in vitro* [77]. Later, in another study [78], some flavonoids including pentagalloylglucose (IC_{50} = 4.5 μM) myricitrin-2″-gallate (IC_{50} = 32 μM) and (-)-epigallocatechin-3-0-gallate (IC_{50} = 52 μM) as the main xanthine oxidase inhibitors along with myricetin (IC_{50} = 3 μM), quercetin (IC_{50} = 5 μM), myricetin-3-O-α-L-arabinopyranoside, myricetin-3-O-β-D-xyloside and myricitrin (IC_{50} of 62.5 – 104 μg/ml) were isolated from the acetone–water extract of the plant.

In a search for xanthine oxidase inhibitors from *Tamus communis* L. (Dioscoreaceae), a popular Algerian folk medicinal plant used for treatment of inflammatory pain such as rheumatism, arthritis, lumbago and dermatosis, it has been demonstrated that the root extracts of this plant is able to inhibit bovine, sheep and human xanthine oxidase activities in a concentration-dependent manner [79]. These inhibitory activities were attributed to the flavonoids and other phenolic contents of the extract. The ethyl acetate extract or rhizome showed the highest inhibitory effects against the bovine (IC_{50} = 0.15 mg/ml), sheep (IC_{50} = 0.04 mg/ml) and human (IC_{50} = 0.09 mg/ml) xanthine oxidase.

Carallia brachiata (Rhizophoraceae) bark is an antipyretic and cardiotonic drug. It has been shown that carallidin (a trimeric proanthocyanidins) and mahuannin A are potent xanthine oxidase inhibitors of the bark with IC_{50} values of 12.9 and 16.0 μM, respectively [80].

Tea (*Camellia sinensis*) is another plant with a high content of flavonoids that has been studied for its effect on xanthine oxidase activity. Catechins and their derivatives are the major polyphenolic constitutions of tea [81]. But the results reported for the inhibitory effects of catechins on xanthine oxidase activity are not consistent. According to some studies [66,68,75,78], these polyphenolic compounds are weak inhibitors of xanthine oxidase or devoid of any inhibitory activity. However, some other studies [65,71] have shown that catechins can act as moderate or potent inhibitor of xanthine oxidase. For example, according to some reports [66,68],

epigallocatechin gallate acts as a weak inhibitor of xanthine oxidase with an IC_{50} value of > 40 μM, whereas other studies have found this compound as a potent competitive inhibit of the enzyme with K_i value of 0.76 μM, which is comparable to that of allopurinol [65]. On the other hand, catechin has been found to be 400 times less effective as xanthine oxidase inhibitor than epigallocatechin gallate [65], but Özyürek *et al.* [71] have reported the inhibitory activities of both catechin and epigallocatechin gallate almost equal to those of quercetin, myricetin, Kaempferol and luteolin. According to Özyürek *et al.* [71], these controversial results may arise from possible oilgomerization of the compound in solutions of reduced water activity.

Artichoke (*Cynara scolymus* L.) which is used for the treatment of hyperuricemia and gout is also a flavonoid-rich plant whose activity against xanthine oxidase has been investigated by Sarawek *et al.* [73]. Although the artichoke leaf extract had very low inhibitory activity on the enzyme, its isolated flavonoids, aglycone luteolin, potently inhibited xanthine oxidase with an IC_{50} value of 1.49 μM [73]. However, the IC_{50} values of luteolin 7-O-glucoside and luteolin 7-O-glucuronide were 19.90 and 20.24 μM, respectively, indicating that the glycoside forms of the luteolin are moderate inhibitors of xanthine oxidase [73]. In spite of these *in vitro* inhibitory activities on xanthine oxidase, Sarawek *et al.* could not find hypouricemic property for the flavonoids after oral administration [73].

In a study carried out by Owen and Johns, 26 species used for the treatment of gout in northeastern North America were assayed for their activity on xanthine oxidase [70]. About 86% of the plant extracts showed inhibitory effects on xanthine oxidase activity, but most of these extracts acted as a relatively weak inhibitor. The highest inhibitory activity was found for *Larix laricina*. Although a positive correlation was obtained between xanthine oxidase inhibitory activity and total phenolic content of plant extract, some phenol-rich plants such as *Melilotus canadense* and *Melilotus alba* displayed low xanthine oxidase inhibitory activity. On the other hand, some species such as *Achillea millefolium* with relatively low phenolic content had high inhibitory effects on the enzyme activity. According to the authors, this could have arisen from a high variation in the possible mechanisms for enzyme inhibition [70]. However, it seems that the presence of high tannin content in the extract along with phenolic compound could cause the problem, as tannins can react nonspecifically with protein enzymes.

In an extensive study, Kong *et al.* studied a total of 122 traditional Chinese plants used for the treatment of gout and other hyperuricemia-related disorders [67]. They found that out of these 122 plants, 69 metanolic and 40 water extracts have inhibitory effects on xanthine oxidase activity at 100 μg/ml. Among the active extracts, 29 metanolic and 13 water extracts possessed greater than 50% inhibition. The most active was found to be the methanolic extract of *Cinnamomum cassia* (Luraceae) twigs (IC_{50} = 18 μg/ml) followed by *Chrysanthemum indicum* (Astraceae) flowers (IC_{50} = 22 μg/ml), *Lycopus europaeus* (Lamiaceae) leaves (IC_{50} = 26 μg/ml) and water extract of *Polygonum cuspidatum* (Polygonaceae) rhizomes (IC_{50} = 38 μg/ml). The IC_{50} value found for allopurinol in this study was 1.06 μg/ml. Based on some published results, most of the observed xanthine oxidase inhibitory activity may be attributed to the polyphenolic compounds present in the active extracts [67].

4.2 Effects of flavonoids on xanthine oxidase activity: *in vivo* studies

The ability of flavonoids to prevent production of some oxidants through inhibition of xanthine oxidase activity could raise the utility of flavonoids as possible therapeutic interventions in those conditions that are accompanied with oxidative stress and generation of ROS such as ischemia–reperfusion injuries, inflammation, cataract and cancer. However, less information is available about the *in vivo* xanthine oxidase function in relation to consumption or clinical application of flavonoids. Most of studies concerning the effects of flavonoid on xanthine oxidase *in vivo* activity have focused on the hypouricemic action of flavonoids.

Biota orientalis (L.) Endl. (Cupressaceae) is a rich source of flavonoids such as quercetin and rutoside and its extract is being used for treatment of gout and rheumatism in Chinese medicine [82]. According to Zhu *et al.* [82], following daily oral administration of *B. orientalis* extract at 100 mg/kg even for 1 day, the urate levels are significantly reduced in oxonate-induced mice. Compared with the administration of 100 mg/kg quercetin, the hypouricemic effect of *B. orientalis* extract was lower, but the extract showed higher hypouricemic activity than its other flavonoid constitute, rutin [82]. Zhu *et al.* showed that the extract, quercetin and rutin have prominent xanthine dehydrogenase/xanthine oxidase inhibitory effect, and the observed hypouricemic effects of *B. orientalis* extract and its flavonoids could be, at least in part, due to their inhibitory effects on xanthine dehydrogenase/xanthine oxidase activities. Interestingly, the reductive effect of the flovonoids administration for three times on urate levels in hypouricemic mice was found to be comparable to that of allopurinol administration at 10 mg/kg [82].

In a recent study, the hypouricemic activities of 15 flavonoids were examined in potassium oxonate-induced hyperuricemic mice [74]. Those flavonoids with high inhibitory effects on xanthine oxidase such as quercetin, myricetin and kaempferol were found to be efficient hypouricemic agents. Those flavonoids such as genistein, daidzin and silibinin that are weak inhibitors of xanthine oxidase activity had no effects on the serum uric acid levels in hyperuricemic mice. However, luteolin and apigenin were exceptions. The hypouricemic property of these two flavonoids was not well correlated with their inhibitory action on xanthine oxidase. Luteolin is a potent inhibitor of xanthine oxidase with an IC_{50} value < 1 μM [66,68]. But in the Mo *et al.* study, this flavonoid showed its significant effects only at a higher dose [74].

Apigenin which is also a potent inhibitor of xanthine oxidase [68,69] could not reduce significantly the liver uric acid levels in hyperuricemic animals even at a higher dose [74]. Analysis of the chemical structure showed that a planar structure with the hydroxyl groups in the position 3, 5 and 7 plays a crucial role in hypouricemic activity of flavonoids [74] which is the case with xanthine oxidase activity [66,68]. Therefore, the study of Mo *et al.* gives further evidence for the hypothesis that the hypouricemic action of some flavonoids in hyperuricemia could be, at least partly, due to their inhibitory effects on xanthine oxidase activity.

Onion (*Allium cepa Lilliaceae*) is a staple food with a high content of flavonoids with quercetin 4′-O-β-glucoside (Q4′G) and quercetin 3,4′-O-β-diglucosides (Q3,4′G) as its two major flavonoids [83]. However, following consumption of an onion meal, both monoglucoside and diglucoside are efficiently hydrolyzed in the small intestine by β-glucosidases to quercetin, most of which is then absorbed [84]. Haidari *et al.* have shown that daily oral administration of either onion juice at 5 g/kg or 5 mg/kg quercetin for 14 days can reduce serum uric levels in hyperuricemic rats in a time-dependent manner with no significant effects on the level of uric acid in the normal animals [85]. The onion juice and quercetin administration also led to a decrease in xanthine oxidase/xanthine dehydrogenase activity and a significant improvement in biomarkers of oxidative stress in hyperuricemic rats. Allopurinol administration at 5 mg/kg reduced the uric acid levels and xanthine oxidoreductase activity more efficiently than onion juice and quercetin, but could not significantly change oxidative stress biomarkers [86]. The extent of the hypouricemic action of onion juice may not be parallel to the change in xanthine oxidoreductase activity [87]. Therefore, it seems that other mechanisms, apart from inhibition of xanthine oxidase activity, are involved in the hypouricemic action of onion juice [87].

In another study similar to the previous mentioned study [85], effects of oral tart cherry juice administration on xanthine oxidase activity in hyperuricemic rats were investigated [86]. Tart cherry (*Prunus cerasus*) contains significant amounts of anthocyanins in addition to other polyphenols such as chlorogenic acid, gallic acid, *p*-coumaric acid and quercetin [88,89]. Its anthocyanins content is estimated to be between 100 and 400 mg of anthocyanins/kilogram of fresh weight [88]. Due to this high content of polyphenolic compounds, tart cherry anthocyanins have been recommended as chemopreventive agents or nutritional supplements [90,91]. As anthocyanins could act as xanthine oxidase inhibitors [92], it might have potential for xanthine oxidase inhibition and hypouricemic property. It has been shown that the oral administration of 5 ml/kg tart juice to potassium oxonate-induced hyperuricemic rats for 2 weeks could significantly reduce the serum uric acid levels in a time-dependent manner [86]. The administration of this dose of the juice had not a significant effect on the serum uric acid concentration in normal rats which could be considered as an advantage for tart juice [86]. Tart cherry juice treatment also inhibited hepatic xanthine oxidase/dehydrogenase activity. The hypouricemic and the inhibitory effects of tart juice were much lower than that of 5 mg/kg allopurinol, but unlike allopurinol, tart juice could also significantly improve serum total antioxidant capacity. Therefore, according to Haidari *et al.* studies [85,86], these features of tart cherry and also onion juice make them attractive candidates for allopurinol, or at least in combination therapy to minimize the side effects of allopurinol, particularly in long-term application [86].

In **Tables 1** and **2**, inhibition data of some pure flavonoids and the extract of flavonoid-containing plants with xanthine oxidase have been summarized**.**

4.3 Effects of flavonoids on aldehyde oxidase activity: *in vitro* studies

Compared with xanthine oxidase, there are extremely limited studies concerning the effects of flavonoids on aldehyde oxidase and the interaction of flavonoids with the latter enzyme has not been covered enough in previous studies compared with the former one. Only in the last few years, some attention has been paid to the influence of flavonoids on aldehyde oxidase activity. In a more recent study, the inhibitory effects of *Ruta graveolens L.* extraction on guinea-pig liver aldehyde oxidase were demonstrated by some authors [93]. *Ruta graveolens* L. (Family: *Rutaceae*) is a flavonoid-rich plant that has been traditionally used as a sedative and antihelmintic to relieve menstrual and gastrointestinal disorders [94,95]. It also has some hypotensive [96] antifertility [97] and anti-inflammatory effects [98]. The most biological properties of *R. graveolens* L. have been attributed to its flavonoids [99,100]. According to Pirouzpanah *et al.* [93], the total extract of *R. graveolens L.* and also its major flavonoids, quercetin and rutin can inhibit guinea-pig hepatic aldehyde oxidase activity. The extract at 100 μg/ml exhibits the same inhibitory effect as 10 μM menadione, a specific potent inhibitor of aldehyde oxidase, on the enzyme activity. This inhibitory effect could be 2 – 11 times higher than those reported for xanthine oxidase [93].

As mentioned before, aldehyde oxidase can metabolize both aldehydes and N-heterocycles and different compounds from these groups of compounds have been used as substrates for the determination of the activity of this enzyme. Interestingly, it has been shown that the inhibitory effects of *R. graveolens L.* flavonoids on aldehyde oxidase could be substrate-dependent and the aldehyde oxidase-catalyzed oxidation of aldehydes monitored by benzaldehyde and vanillin is inhibited more potently than N-heterocycle oxidation measured by phenanthridine [93]. The IC_{50} values for the inhibitory effect of *R. graveolens L.* extract against the oxidation of guinea-pig liver aldehyde oxidase-catayzed oxidations of benzaldehyde, vanillin and phenanthridine have been reported to be 10.4, 10.1 and 43.2 μg/ml, respectively [93]. The same manner was

Table 1. Inhibition of xanthine oxidase by flavonoid-rich extracts from various medicinal plants.

Plant	Plant part	Inhibition parameter	Ref.
Achillea millefolium (Astraceae)	Aerial part	Inhibition (%) at 100 μg/ml = 66.64*	[70]
Artemisia anomala (Astraceae)	Whole plant	IC_{50} = 36 μg/ml* IC_{50} = 66 μg/ml‡	[67]
Astragalus membranaceus (Fabaceae)	Root	IC_{50} = 85 μg/ml*	[67]
Carthamus tinctorius (Astraceae)	Fruit	IC_{50} = 64 μg/ml*	[67]
Chrysanthemum indicum (Astraceae)	Flower	IC_{50} = 22 μg/ml* IC_{50} = 91 μg/ml‡	[67]
Cinnamomum cassia (Luraceae)	Twig	IC_{50} = 18 μg/ml* IC_{50} = 40 μg/ml‡	[67]
Cinnamomum cassia (Luraceae)	Bark	IC_{50} = 58 μg/ml* IC_{50} = 78 μg/ml‡	[67]
Citrus sinensis (Rutaceae)	Fruit shell	IC_{50} = 98 μg/ml*	[67]
Cuscuta chinensis (Convolvulaceae)	Seed	IC_{50} = 53 μg/ml*	[67]
Erythrina indica (Fabaceae)	Bark	IC_{50} = 70μg/ml*	[67]
Fraxinus rhynchophylla (Oleaceae)	Bark	IC_{50} = 28 μg/ml* IC_{50} = 53 μg/ml‡	[67]
Glechoma longituba (Lamiaceae)	Whole plant	IC_{50} = 48 μg/ml*	[67]
Gleditsia sinensis (Fabaceae)	Spike	IC_{50} = 106 μg/ml* IC_{50} = 108 μg/ml‡	[67]
Kochia scoparia (Chenopodiaceae)	Seed	IC_{50} 116 μg/ml‡	[67]
Larix laricina (Pinacea)	Inner bark	Inhibition (%) at 100 μg/ml = 86.3*	[70]
Ligusticum brachylobum (Apiaceae)	Rhizome	IC_{50} = 34 μg/ml*	[67]
Lycopodium calvatum (Lycopodiacea)	Whole plant	IC_{50} = 94 μg/ml*	[67]
Lycopus europaeus (Lamiaceae)	Leaf	IC_{50} = 26 μg/ml*	[67]
Lycopus europaeus (Lamiaceae)	–	IC_{50} = 79 μg/ml* IC_{50} = 26 μg/ml‡	[67]
Morus alba (Moraceae)	Twig	IC_{50} = 57 μg/ml*	[67]
Morus alba (Moraceae)	Bark	IC_{50} = 71 μg/ml*	[67]
Origanum majorana (Lamiacea)	Aerial part	IC_{50} = 7.71 μg/ml	[71]
Polygonum cuspidatum (Polygonaceae)	Rhizome	IC_{50} = 38 μg/ml	[67]
Populus balsamifera (Salicaceae)	Inner bark	Inhibition (%) at 100 μg/ml = 51.29*	[70]
Prunella vulgaris (Lamiaceae)	Whole plant	IC_{50} = 86 μg/ml*	[67]
Salvia miltiorrhiza (Lamiaceae)	Root	IC_{50} = 96 μg/ml‡	[67]
Salvia officinalis (Lamiaceae)	Leaf	IC_{50} = 7.95 μg/ml	[71]
Scutellaria barbata (Lamiaceae)	Whole plant	IC_{50} = 46 μg/ml* IC_{50} = 66 μg/ml‡	[67]
Smilax china (Liliaceae)	Rhizome	IC_{50} = 62μg/ml*	[67]
Smilax glabra (Liliaceae)	Rhizome	IC_{50} = 33 μg/ml* IC_{50} = 96 μg/ml‡	[67]
Tamus communis (Dioscoreaceae)	Rhizome	IC_{50} = 40 – 150 μg/ml§	[79]
Trachelospermum jasminoides (Apocynaceae)	Stem	IC_{50} = 108 μg/ml* IC_{50} = 113 μg/ml‡	[67]

*Methanol extract.
‡Water extract.
§Ethyl acetate extract.

Table 2. Inhibition parameters for the interaction of some flavonoids with xanthine oxidase.

	IC_{50} (μM)	Inhibition constant (μM)	Ref.
Flavonol			
Myricetin	0.7	–	[58]
	1.5	–	[68]
	1.72	–	[71]
	3	–	[78]
	–	K_i = 2.17	[69]
Myricitrin-2″-gallate	32	K_i = 32	[78]
Quercetin	0.4	–	[58]
	0.44	K_i = 0.28	[66]
	1.5	–	[68]
	2.08	–	[70]
	5	–	[78]
	–	K_i = 1.40	[69]
	7.23	K_i = 0.25 (mixed-type)	[65]
Kaempferol	0.67	K_i = 0.23	[66]
	2.28	–	[71]
	2.5	–	[68]
	–	K_i = 0.33 (mixed-type)	[65]
Rhamnetin	> 50	–	[66]
Isorhamnetin	0.4	K_i = 0.17	[66]
Tangeretin	> 100	–	[66]
Rutin	30.1	–	[58]
	46.8	–	[66]
Galagin	4	–	[68]
Fisetin	11.3	–	[68]
Hyperoside	35	–	[58]
Morin	3.7	–	[68]
	6.9	–	[58]
Robinetin	9	–	[68]
Resokaempferol	22	–	[68]
Flavone			
Apigenin	1	–	[68]
	1.5	–	[71]
	–	K_i = 0.61	[69]
Luteolin	0.75	–	[68]
	0.96	K_i = 0.31	[66]
	1.49	–	[73]
	2.38	–	[71]
Luteolin-7-O-glucoside	19.90	–	[73]
Luteolin-7-O-glucuronide	20.24	–	[73]
Chrysin	2.5	–	[68]
	5.02	–	[66]
Isovitexin	–	K_i = 5.22	[69]
Flavanol			

Table 2. Inhibition parameters for the interaction of some flavonoids with xanthine oxidase (continued).

	IC_{50} (μM)	Inhibition constant (μM)	Ref.
(+)-Catechin	1.93	–	[71]
	2.5	–	[78]
	70.9	–	[58]
	–	K_i = 303.95 (un-competitive)	[65]
	No inhibition	–	[66]
(-)-Epicatechin	2.04	–	[71]
	59.2	–	[58]
	> 40	–	[68]
	No inhibition	–	[66]
	–	K_i = 20.48 (mixed-type)	[65]
Epigallocatechin	1.85	–	[71]
	> 100	–	[66]
	–	K_i = 10.66 (mixed-type)	[65]
Epigallocatechin gallate	1.75	–	[71]
	> 40	–	[68]
	44.7	–	[66]
	52	K_i = 31	[78]
	–	K_i = 0.76	[65]
Epicatechin gallate	1.71	–	[71]
	48.5	–	[66]
	–	K_i = 2.86 (mixed-type)	[65]
Carallidin	12.9	–	[80]
Mahuannin A	16	–	[80]
Flavanone			
Naringenin	> 50	–	[69]
Naringin	49.40	–	[71]
	> 100	–	[58]
Hesperetin	26.6	–	[58]
	27.4	–	[66]
Hesperidin	> 100	–	[58]
Liquritigenin	49.3	K_I = 151.6, K_i = 14	[76]
Flavanonol			
(±)-Taxifolin	28.5	–	[58]
	> 40	–	[68]
	> 100	–	[66]
Silybin	12	K_I = 100, K_i = 9.22	[101]
Chalcone			
Isoliquiritigenin	55.8	K_I = 81.9, K_i = 17.4	[76]
Isoflavone			
Daidzein	> 40	–	[68]
	> 100	–	[66]
Genistein	> 40	–	[68]
	83	–	[66]
	–	K_i = 3.23	[69]
Allopurinol (the putative xanthine oxidase inhibitor)	–	K_i = 0.34	[69]
	0.6	–	[78]
	3.2	–	[58]
	6.2	–	[68]

found for quercetin and rutin isolated from the extract. This has been attributed to the involvement of different isoforms of aldehyde oxidase or different interactions of flavonoids with aldehyde and N-heterocycle oxidations catalyzed by hepatic aldehyde oxidase [93].

Milk thistle (*Silybum marianum* L.) is another flavonoid-rich plant whose effect on aldehyde oxidase has been recently studied [101]. *Silybum marianum* L. has been widely used around the world for > 2000 years as a dietary supplement and also for its strong antihepatotoxic efficacy [102,103]. It also possesses antifibrotic, anti-inflammatory, immunomodulating and other activities [102,104,105]. The active constituent of silybum marianum is silymarin which is a mixture of flavanolignans (silibinin, isosilybin, silychristin, silydianin). Silibinin is the major and most active component in silymarin representing about 60 – 70% of silymarin [106]. Silymarin is mainly extracted from the seeds of the milk thistle, although it can also be found in trace amounts in other parts of the plant [107,108]. The effects of silibinin on different enzymes have been investigated. According to Wu *et al.* [108], silibinin is a weak inhibitor (IC_{50} > 200 μM) of cytochrome P450 isozymes. Unlike cytochrome P450 system, both molybdenum hydroxylases are strongly inhibited by silibinin and the seed extract of *S. marianum* L., although the inhibitory effects of the extract and its flavanolignan on xanthine oxidase activity are slightly higher than those on aldehyde oxidase activity [101]. The IC_{50} values of the seed extract of *S. marianum* L. on rat liver xanthine oxidase and aldehyde oxidase are 17 and 39 μg/ml, respectively. The corresponding values for silibinin are 12 and 38 μM, respectively [101].

More recently, in a comparative study, the effects of twelve flavonoids from three subclasses of flavonoids (flavonol, flavanol and flavanone) on guinea-pig liver aldehyde oxidase (measured by vanillin and phenanthridine as substrates) and xanthine oxidase (measured by xanthine as substrate) activities were investigated by Pirouzpanah *et al.* [58]. Among the selected flavonoids, quercetin and myricetin were found as the most potent inhibitors of aldehyde oxidase and xanthine oxidase. These inhibitory effects were comparable to those of menadione and allopurinol, the putative inhibitors of aldehyde oxidase and xanthine oxidase, respectively. In general, with all three subclasses, aldehyde oxidase activity was inhibited more effectively than xanthine oxidase activity. This was more significant with flavanol and flavanone subclasses compared with the flavonol subset [58]. Almost all flavanoids examined in the Pirouzpanah *et al.* study caused more inhibition on the aldehyde oxidase activity towards vanillin, as an aldehyde substrate of aldehyde oxidase, than phenanthridine, the N-heterocyclic substrate of the enzyme. It appears that aldehyde oxidase-catalyzed oxidation of aldehydes is influenced in different ways by flavonoids compared with the N-heterocycles oxidation. In **Table 3**, the inhibition data of some flavonoids on aldehyde oxidase activity have been summarized.

4.4 Effects of flavonoids on aldehyde oxidase activity: *in vivo* studies

Unlike the numerous studies on xanthine oxidase activity, the number of *in vivo* studies on aldehyde oxidase activity has been very limited. The increased studies on *in vivo* xanthine oxidase activity mainly rely on the availability of a simple and reliable method for the evaluation of the enzyme activity *in vivo* based on the measurement of uric acid production from xanthine oxidation. However, in the case of aldehyde oxidase, in spite of the existence of a wide range of substrate for this enzyme, none of these compounds are completely appropriate for the determination of the enzyme activity *in vivo*. Therefore, the study of aldehyde oxidase activity *in vivo* and the effects of different compounds on the enzyme activity have not received enough attention. This is the case with the effects of flavonoids on *in vivo* aldehyde oxidase activity and unlike the other molybdenum hydroxylase, xanthine oxidase, no results are available in this area. Recently, aldehyde oxidase activity *in vivo* has been estimated through examination of *in vivo* conversion ratio of N^1-methylnicotinamide to N^1-methyl-2-pyridone-5-carboxamide and N^1-methyl-4-pyridone-5-carboxamide [109]. Production of 7-hydroxymethotrexate from methotrexate and penciclovir from famcilovir are also catalyzed by aldehyde oxidase that have the potential to be used for the evaluation of the enzyme activity *in vivo* [51,56,110]. Development of a valid method based on the above reactions would provide a broad area to study *in vivo* aldehyde xoiadse activity.

4.5 Structure–activity relationship studies of flavonoids as inhibitors of molybdenum hydroxylases activity

The flavonoids with flavone (e.g., luteolin) and flavonol (e.g., quercetin and myricetin) structures show marked inhibition on xanthine oxidase and aldehyde oxidase activities; however, reduction of C2-C3 double bond in the C ring of either flavone (e.g., hesperetin and naringenin) or flavonol (e.g., taxifolin) backbone results in a relatively marked reduction in the inhibitory effects of flavonoids on xanthine oxidase but not aldehyde oxidase activities indicating the importance of planar structure for xanthine oxidase inhibition [66,68,69]. It appears that this is not crucial for inhibition of aldehyde oxidase activity, although flavonoids with planar structure are relatively more potent inhibitors of aldehyde oxidase activity than the corresponding flavonoids with non-planar structure [58].

Existence of a hydroxyl group at 3-position of the flavonoid structure is not essential for the inhibitory activity of the compound against molybdenum hydroxylases, although it may cause some increase [58,66] or decrease [68] in the inhibitory effects of the flavones on the enzyme activity. However, substitution of this hydroxyl group either in flavonol or in flavanone backbone leads to a significant reduction in the inhibitory capacity of the flavonoid on both xanthine oxidase [65,68] and aldehyde oxidase activities [93].

Table 3. Inhibitory effects of some flavonoids on aldehyde oxidase activity towards aldehyde (measured by vanillin as the substrate) and N-heterocyclic (measured by phenanthridine as the substrate) compounds [58,101].

Flavonoids	Aldehyde substrate		N-Heterocyclic substrate	
	IC_{50} (μM)	Inhibition constant (μM)	IC_{50} (μM)	Inhibition constant (μM)
Flavonol				
Myricetin	0.4	K_i = 0.3, K_i = 0.7	0.7	K_i = 2.5, K_i = 0.7
Quercetin	0.4	K_i = 0.4, K_i = 0.5	2.1	K_i = 1.7, K_i = 0.8
Rutin	17.8	K_i = 25.5, K_i = 12.5	22.2	K_i = 27.4, K_i = 13.9
Hyperoside	20.8	K_i = 27.8, K_i = 15.8	24.3	K_i = 17.6, K_i = 4.7
Morin	3.7	K_i=5.0, K_i = 4.6	30.1	K_i = 18.5, K_i = 17.3
Flavanol				
(+)-Catechin	2.5	K_i = 2.3	6.1	K_i = 1.3
(-)-Epicatechin	5.7	K_i = 4.2	6.1	K_i = 2.4
Flavanone				
Naringenin	3.4	K_i = 2.0	8.8	K_i = 3.9, K_i = 1.6
Naringin	100<		100<	
Hesperetin	4.3	K_i = 2.6	9.8	K_i = 4.5, K_i = 1.9
Hesperidin	100<		100<	
Flavanonol				
(±)-Taxifolin	4.1	K_i = 3.4	14.7	K_i = 10.0, K_i = 4.1
Silybin	-	-	38	K_i = 29.5

Accordingly, quercetin as a flavonol with a free hydroxyl group at C-3 is a potent inhibitor of xanthine oxidase and aldehyde oxidase with IC_{50} values around 1 μM, whereas its glycosylated forms, rutin and hyperoside are moderate inhibitors of both enzymes (IC_{50} > 80 μM). Similarly, in the flavanone subclasses, the IC_{50} values of hesperidin and naringin are much higher than those of hesperetin and naringenin which are the glycosylated forms of two latter flavonoids, respectively (**Table 4**). Similar results have been reported for structure–activity relationship of steroids and aldehyde oxidase. It has been shown that substitution of hydroxyl group at C-3′ of A ring in β-stradiol reduces the inhibitory effect of the steroid on aldehyde oxidase activity [111,112]. According to some authors, the reduction in the xanthine oxidase activity could be explained by the destabilization of the polar hydroxyl stretching into the hydrophobic region of active site of the enzyme and lowering binding affinity [69].

Flavonoids as polyphenolic compounds have several hydroxyl groups in their structure. The number and location of these groups have prominent effect on different inhibitory properties of flavonoids. However, the latter property seems to play a more important role than the former one. The largest contribution results from the presence of a hydroxyl group at 5 and/or 7 locations. Because 7-hydroxyflavone is the only flavone with one hydroxyl group that shows measurable inhibitory effect on xanthine oxidase activity [68], this chemotype is relatively more important than the 5-hydroxyl moiety in the inhibition of the enzyme activity. Van Hoorn *et al.* [68] have provided several examples indicating that introduction of an extra hydroxyl group at C-5 alongside 7-hydroxyl group increases xanthine oxidase inhibitory capacity of the flavonoid by a factor of 5.3 – 16 compared with those analogues that have only 7-hydroxyl group. Masking these hydroxyl groups may result in a dramatic effect on the inhibitory activity of the flavonoid. For instance, the IC_{50} value of rhamnetin, a 7-methoxy derivative of quercetin, for inhibition of xanthine oxidase could be > 100 times higher than that of quercetin [66]. These results indicate the importance of 7-hydroxyl group, particularly in combination with the 5-hydroxyl group, in inhibition of xanthine oxidase. Unfortunately, the independent effect of 5-position on xanthine oxidase inhibition cannot be deduced from the reported data and it requires the synthesis of more analogues with the required structural parameters. It is much more difficult to judge the influence of 5 and 7-position of ring A of flavonoids on aldehyde oxidase activity due to the lack of information.

The addition of extra hydroxyl groups at C-3′ and C-4′ positions of B ring of the flavone backbone may slightly increase the inhibitory effect of the flavonoid. This can be concluded from comparing the inhibitory capacity of chrysin, apigenin and luteolin against xanthine oxidase activity [66,68]. But the presence of two hydroxyls at C-5 and C-7-positions is more important than that of C-3′ and C-4′ when chrysin is compared with the analogue that has 3′- and 4′-hydroxyl groups in place of 5- and 7-hydroxyl substituents [68]. There is no clear manner regarding the influence of the presence of extra hydroxyl groups at C-3′, C-4′ and C-5′ positions of the

Table 4. Structural–activity relationships comparison of flavonoids-xanthine oxidase and aldehyde oxidase activities [58,65,66,68,69,93].

	Xanthine oxidase	Aldehyde oxidase
C-2-C-3 double bond	Presence of the double bond (a planar structure) is important for inhibitory activity	The presence of the double bond is not essential; however, flavonoids with planar structure are relatively more potent inhibitor
C-3-OH	It is not essential But, substitution of 3-OH decreases the inhibitory activity	It is not essential But, substitution of 3-OH decreases the inhibitory activity
C-5-OH	Its independent effect is not clear; but along with 7-OH seems to increase the inhibitory activity	There is not enough information to evaluate its effect, but the same results may be applicable with this enzyme
C-7-OH	The most important group in the flavonoids structure Its inhibitory effect is attenuated with 5-OH group	There is not enough information to evaluate its effect, but the same results may be applicable with this enzyme
C-6-OH	Negative contribution to the inhibition activity; but more studies are required	Lack of information
C-8-OH	Negative contribution to the inhibition activity; but more studies are required	Lack of information
C-2′-OH	Negative contribution to the inhibition activity; but more studies are required	Lack of information
C-3′-OH	Slight increase in the inhibitory effect of the flavones but no clear understanding with respect to flavonols	Lack of information
C-4′-OH	Slight increase in the inhibitory effect of the flavones but no clear understanding with respect to flavonols	Lack of information

flavonol structure on the inhibitory effects against molybdenum hydroxylases. All three flavonols, kaempferol, quercetin and myricetin, have almost similar IC_{50} values for inhibition of xanthine oxidase [66,68]. The lack of a significant difference in the inhibitory effects of quercetin and myricetin against guinea-pig hepatic aldehyde oxidase and xanthine oxidase has also been reported by some other investigators [58]. Therefore, the published results [58,66,68] are inconsistent and also insufficient to obtain a clear and documented interpretation regarding the contribution of hydroxyl groups at C-3′, C-4′ and C-5′ positions of flavonols in the inhibitory capacity against molybdenum hydroxylases.

According to Van Hoorn *et al.* [68], the presence of a hydroxyl group at C-2′ reduces the inhibitory effect of flavonoid on xanthine oxidase activity possibly through disruption of hydrogen bonds within the allosteric center due to steric hindrance. This has been concluded from comparing kaempferol with morin and there is no more information to evaluate this hypothesis.

5. Expert opinion and conclusion

Molybdenum hydroxylases, aldehyde oxidase and xanthine oxidase, are metalloflavoproteins that are involved in some physiological and pathophysiological processes, particularly in the metabolism of a wide range of xenobiotics, especially in some important drugs such as famciclovir, acyclovir, 6-mercaptopurine, azathioprine and methotrexate. Therefore, the interference with their functions should be of great importance. Flavonoids are one of the most widespread classes of natural compounds that can interfere with both xanthine oxidase and aldehyde oxidase activities. A part of the importance of this interference could arise from the physiological and biological functions of molybdenum hydroxylases such as purine metabolism, vision and synaptic transmission [40,63,113,114]. As the oxidative reactions catalyzed by xanthine oxidase and aldehyde oxidase lead to generation of ROS, both enzymes have the potential to play an important role in those disorders that ROS has significant contribution.

Therefore, taking into account that flavonoids are found in high content in our daily foods, the inhibition of molybdenum hydroxylases by flavonoids may alter some relevant physiological and pathophysiological processes. However, only few reports are available on molybdenum hydroxylases–flavonoids interactions *in vivo*, and hyperuricemia and gout have been almost the only cases to be considered in the literature with respect to this topic. It appears that the study of flavonoid effects on physiological and pathophysiological processes in which molybdenum hydroxylases play an important role offers a great potential for future studies, and more studies are required to clarify the contribution of molybdenum hydroxylases to the beneficial effect of flavonoids in ROS-associated diseases such as ischemia-reperfusion injury.

Based on the existing data, the importance of molybdenum hydroxylases in drug metabolism outweighs their physiological and pathophysyiological roles. According to some authors, xanthine oxidase and aldehyde oxidase are mostly involved in the detoxification of a wide range of xenobiotics rather than physiological compounds [46]. Compared to cytochrome P450 family, the number of drugs metabolized by molybdenum hydroxylases is few, but it seems that the coming years would witness more drugs cleared by these two enzymes, in particular aldehyde oxidase [115], and there would be more chance for drug–drug interactions that occur via interaction with molybdenum hydroxylases activity. Some of the flavonoids such as myricetin and quercetin are potent inhibitors of molybdenum hydroxylases with K_i and IC_{50} values < 1 μM. These inhibitory effects are comparable with those of other well-known potent inhibitors of these enzymes such as allopurinol (xanthine oxidase inhibitor: K_i = 0.34 μM) [69], menadione (aldehyde oxidase inhibitor: IC_{50} = 0.20 μM) [116], chlorpromazine (aldehyde oxidase inhibitor: K_i = 0.6 μM) [117], raloxifene (aldehyde oxidase inhibitor: IC_{50} = 0.003 μM) [116] and perphenazine (aldehyde oxidase inhibitor: IC_{50} = 0.033 μM) [116].

In spite of these potent inhibitory effects of a large number of flavonoids on molybdenum hydroxylases activity, the interaction of flavonoids with the metabolism of those drugs metabolized by aldehyde oxidase and xanthine oxidase has not received enough attention. Flavonoids are almost an inevitable part of our dietary program and are expected to be consumed in high content in our daily life. Thus, it is more likely that they are ingested in combination with drugs including those that are metabolized by molybdenum hydroxylases. As a result, occurrence of some adverse effects following uncontrolled intake of flavonoids could be a serious concern for consumer safety [18]. Thus, the study of flavonoid interaction with the drugs metabolized by molybdenym hydroxylases is a broad area for scientific studies that has not been covered so far.

As discussed in the present manuscript, the number of studies on the effects of flavonoids, either in the form of dietary supplement or plant extract, on xanthine oxidase activity is much higher than the number of studies on aldehyde oxidase. Bearing in mind the extreme similarity of these two molybdenum containing enzymes, it is the opinion of the authors that those flavonoid-rich plants or pure flavonoids that have been subject for falvonoid–xanthine oxidase interaction studies are more likely to be good candidates for aldehyde oxidase. Meanwhile, based on the limited studies available, the existence of some differences in the interactions of flavonoids with xanthine oxidase and aldehyde oxidase cannot be ruled out and elucidation of these differences can be a challenging subject for the future studies. This is especially applicable for aldehyde oxidase which has wider substrate specificity. More recently, the difference in the manner of the interaction of some flavonoids with different types of aldehyde oxidase substrates has been reported [58]. This approach is being followed by the authors at the present and the results have been very satisfactory.

But, in studying the interaction of flavonoids with any enzyme system including molybdenum hydroxylases, it is very important to consider the pharmacokinetics of flavonoids. Due to some factors such as food processing procedure, life style and food habit, the daily intake of flavonoids may be variable in different communities and different people. Furthermore, flavonoids such as other drugs that are administrated through oral route have to be absorbed before exerting their pharmacological effects. The exact mechanisms of gastrointestinal absorption of polyphenols are not completely understood. Dietary flavonoids mainly occur as glycosides which may possess different kind and number of sugars. Investigations have shown that aglycones are the preferred forms of flavonoids to be absorbed; therefore, it would be necessary for glycosides to be hydrolyzed before absorption. The nature of sugar residue has a determinant influence on the absorption of the respective aglycone moiety [18]. After absorption, the aglycones are conjugated and each of these conjugated metabolites may have different biological effects. Therefore, the metabolic pathway of each flavonoid should be separately considered in the evaluation of the flavonoid interactions with molybdenum hydroxylases functions. This would open another area for scientific study in the coming years.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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