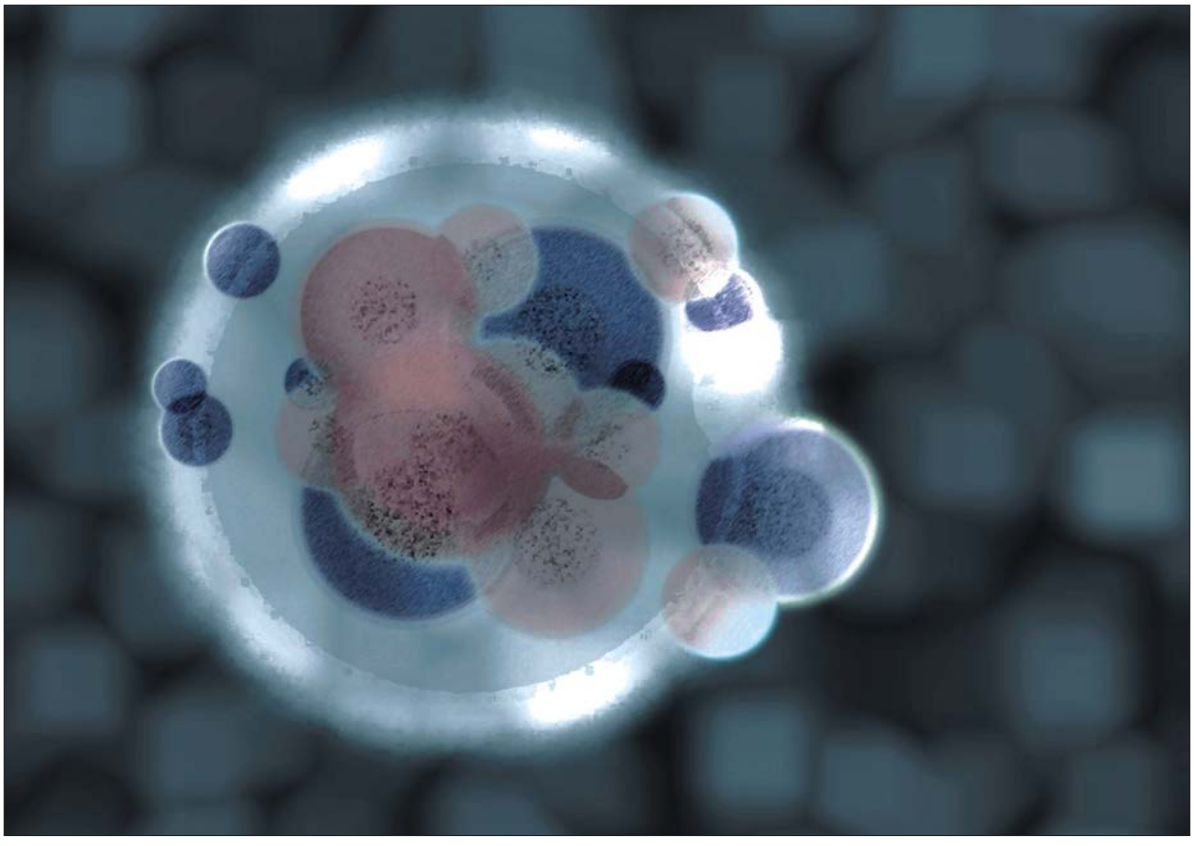

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Comparative Effects of Scopoletin and Menadione on Aldehyde Oxidase Activity of Guinea Pig Liver

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Abstract: Scopoletin, 7-hydroxy-5-methoxycoumarin, has been found to be a moderate inhibitor for aldehyde oxidase, a molybdo-flavoenzymes. It inhibits the release of superoxide anion more efficiently than hydrogen peroxide or substrate oxidation. Statistically, the inhibition of three-specific aldehyde oxidase substrates was significant ($p<0.005$) in the presence of 100 μM Scopoletin. A progressive and non-competitive inhibition has been observed with inhibitor constant (K_i) value of 57.2±3.3 μM using indole-3-aldehyde and oxygen as a substrate and electron acceptor, respectively. A specific site for interaction between Scopoletin and aldehyde oxidase has been proposed and its effect on the reactive oxygen species production has been discussed. The effect of Scopoletin on aldehyde oxidase activity has been compared with that of traditional and specific inhibitors, such as menadione.

Key words: 7-hydroxy-5-methoxycoumarin, Scopoletin, menadione, molybdenum hydroxylase, hydrogen peroxide, superoxide anion

INTRODUCTION

Scopoletin, 7-hydroxy-5-methoxycoumarin, is a naturally occurring component in cotton leaf and citrus peel (Fig. 1). It is a dye that can be used to detect the release of reactive oxygen species during the oxidative burst, peroxynitrite scavenger and as acetylcholinesterase inhibitor[1-3]. Scopoletin and other coumarin compounds has been isolated from cassava, a shrubby tropical American plant (*Manihot esculenta*) widely grown for its large, tuberous, starchy roots and this root is eaten as a staple food in the tropics only after leaching and drying to remove cyanide. The consumption of this plant has been linked to some of the diseases included endemic goiter, cretinism mental retardation, slowly developing tropical neuropathy characterized by optic atrophy, nerve deafness, ataxia, scrotal dermatitis, stomatitis and glossitis[4]. Experimental evidence strongly suggest a biochemical and/or toxicological role for Scopoletin[4]. In fact, cassava roots form the important staple food for more than 500-million people widely spread in the tropical countries such as Asia, Africa and Latin America[5]. In view of both the high human daily intake and the wide spread of cassava diet[4,5], it is important to investigate the biological effect of Scopoletin on aldehyde oxidase metabolizing system.

Aldehyde oxidase (EC 1.2.3.1) one of highly related molybdo-flavoenzymes acting upon a variety of compounds of industrial and medical importance, which catalyzes the oxidation of aldehydes and *N*-heterocyclic compounds to the corresponding carbolxilic acids and lactams, respectively and belong to the xanthine oxidase family[6-8]. Aldehyde oxidase generates superoxide anion and hydrogen peroxide but in contrast to xanthine oxidase (EC 1.1.3.22), aldehyde oxidase seems to be a permanent oxidase, with no activity towards NAD^+. Studies on aldehyde oxidase and xanthine oxidase have shown that modulation of enzyme activities, cofactor availability, substrate concentration and oxygen tension all affect rates of intracellular reactive oxygen species production[6-8]. Consequently, aldehyde oxidase has been implicated in pathophysiology of alcohol liver injury, visual processes, synthesis of retinoic acid and reperfusion tissue injury[9]. Although xanthine oxidase generates reactive oxygen species, it should be noted that *in vivo*, the enzyme exists predominantly as dehydrogenase (EC 1.1.1.204), reacting with NAD^+, whereas aldehyde oxidase reacts exclusively with

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oxygen[10]. Guinea pig liver aldehyde oxidase has been shown to be an excellent model for the human liver enzyme, therefore it has been used throughout this study[8,11]. The aim of the present study was to investigate the interaction of Scopoletin with partially purified guinea pig liver aldehyde oxidase in term of superoxide amion production, hydrogen peroxide formation and the overall substrate oxidation. One of the objectives of this study was to shed more light on the role of molybdenum hydroxylases in toxicity of Scopoletin.

MATERIALS AND METHODS

Instrumentation: Enzyme activity was determined spectrophotometrically using a Cary 50 UV/VIS spectrophotometer (Varian Australia Pty Ltd., Mulgrave/Victoria, Australia), which was linked to a cell temperature control unit. With the exception of enzyme, which was kept in ice until mixing with other components, all solutions were pre-warmed to 37°C. The spectrophotometer was computer-controlled by Carry WinUV® spectroscopy software package with additional kinetics software (2002).

Reagents and chemicals: All reagents and solvents are of analytical grade. All chemicals and reagents were purchased from Sigma-Aldrich Chemical Company Ltd. (Gillingham-Dorset, SP8 4XT, UK). Scopoletin and hydrogen peroxide-30% (w/w) have been purchased from Sigma-Aldrich Chemical Company Ltd (Louis, MO 633178, USA). Cytochrome c (from horse heart), horseradish peroxidase (HRP, type VI) and superoxide dismutase (SOD, from bovine liver suspension in 3.8 M $(NH_4)_2SO_4$, pH 7.0) were also purchased from Sigma Chemical Company Ltd. (Gillingham-Dorset, SP8 4XT, UK).

Animals care: Dunkin-Hartley guinea pigs (600-700 g) were inbred and housed in pairs in plastic cages under a cycle of 07:00-21:00 h light and 21:00-07:00 h dark daily. They were given free access to a diet of FD1 guinea pig pellets supplied by special diet services, hay, twice weekly and water *ad libitum*. They were maintained at a temperature of 18-19°C and humidity at 50%. The animals were killed by cervical dislocation between 10:00 am and 11:00 am daily.

Sorenson's phosphate buffer (67 mM), pH 7.0: Sorenson's phosphate buffer (67 mM) was made from two separate solutions containing either 9.511 g L^{-1} Na_2HPO_4 or 9.118 g L^{-1} KH_2PO_4 in distilled water. The buffer was made by adding 611 mL of Na_2HPO_4 solution to 389 mL of KH_2PO_4 solution and the pH was adjusted to pH 7.0 with 0.3 M phosphoric acid, using a pH-meter. EDTA (0.0372 g) was added to one liter of above mixture to give a final concentration of 100 μM EDTA. The buffer was stable for at least one month at 4°C.

Preparation of partially purified molybdenum hydroxylases from guinea pig liver: Aldehyde oxidase was partially purified from liver homogenate of mature Dunkin-Hartley guinea pigs following a published methodology[11]. Partially purified enzyme was stored in liquid N_2 until needed.

Determination of initial oxidation rates and production of superoxide anion: Aldehyde oxidase activity in partially purified molybdenum hydroxylase fractions was monitored, at 37°C, using 100 μM 2-pyrimidinone, 50 μM phenanthridine, 100 μM phthalazine (enzyme fraction was diluted, 1:10) and 100 μM indole-3-aldehyde (1:40 dilution) as substrates in 67 mM Sorenson's phosphate buffer, pH 7.0, containing 100 μM EDTA. Enzyme activity of guinea pig liver molybdenum hydroxylase fractions was also measured in the presence of 1, 10, 50 and 100 μM Scopoletin. The initial velocity for substrate oxidation was determined by measuring the change in absorbance/minute and calculating enzyme activities in μMol/min/mg protein in the presence and absence of Scopoletin. The following equation was used to calculate the enzyme activity:

$$\text{Initial rate of substrate oxidation (}\mu\text{Mol/min/mgprotein)} = \frac{\text{[Absorbance change / minute] x 1000}}{\text{[Molar absorptivity] x [Protein concentration in cuvette (mg/ml)]}}$$

Reduction of cytochrome c by partially purified molybdenum hydroxylase fractions at 550 nm was followed using phthalazine, indole-3-aldehyde, 2-pyrimidinone, phenanthridine and xanthine as substrates. Superoxide amion reacts with ferricytochrome c reducing Fe(III) to Fe(II).

Fluorimetric determination of hydrogen peroxide formation: Hydrogen peroxide formation was monitored fluorimetrically, using an MPF-3 Fluorescence Spectrophotometer with the excitation wavelength set at 395 nm and the emission wavelength set at 470 nm. The slit width was fixed at 6 mm for both excitation and emission, using a 150-watt Xenon Lamp as the light source. Hydrogen peroxide generated during molybdenum hydroxylase-catalysed oxidation was reacted with horseradish peroxidase to form a complex (compound I). The complex causes the oxidation of the fluorophore,

Scopoletin, resulting in a decrease in fluorescence, which is proportional to original hydrogen peroxide content as described in detail previously[12].

Protein determination for partially purified molybdenum hydroxylase fractions: A Pierce Bicinchoninic acid (BCA®) protein reagent assay kit was used. This technique depends on the production of Cu (I) from the reaction of protein with Cu (II) in an alkaline medium as described by Smith *et al.*[13].

Statistical analysis: Means were compared using student t-test and the level of significant difference was determined at $p<0.05$-$p<0.005$.

RESULTS AND DISCUSSION

In this study, Scopoletin inhibited the oxidation of phthalazine, indole-3-aldehyde and 2-pyrimidinone catalyzed by aldehyde oxidase. However, it would appear that Scopoletin is a progressive inhibitor of aldehyde oxidase as inhibition was more marked as the reaction proceeded. Aldehyde oxidase and xanthine oxidase are both present in guinea pig partially purified molybdenum hydroxylase fractions; accordingly specific enzyme inhibitors were used to confirm the specificity of the spectrophotometric assay. Chlorpromazine and menadione were used as specific aldehyde oxidase inhibitors[8,14]. Under the conditions used in this study, these inhibitors usually inhibit substrate oxidation by 98-99%[14]. In the present study, 100 μM of chlorpromazine and menadione decreased initial oxidation rates of 100 μM phthalazine, indole-3-aldehyde, 2-pyrimidinone and 50 μM phenanthridine by 95-98% ($p<0.001$). In contrast, 100 μM allopurinol, a xanthine oxidase inhibitor, caused a negligible reduction in substrate oxidation (1-2%)[15]. As it has been shown that allopurinol is slowly converted by aldehyde oxidase to oxipurinol, it though that allopurinol is a competitive substrate of aldehyde oxidase[14].

Scopoletin was tested as an inhibitor for aldehyde oxidase activity and found to be a moderate, but significant, inhibitor (Fig. 2). The effect of Scopoletin on substrate oxidation has been compared to classical aldehyde oxidase inhibitors (Table 1). By using 1-tailed student's t-test, it has been shown that 100 μM Scopoletin caused a significant inhibition ($p<0.005$).

Scopoletin was incubated with the enzyme preparation using oxygen as electron acceptor and the incubation mixture was monitored by repetitive scanning between 200-700 nm for up to 10 min. There were no change observed in the spectrum of Scopoletin. It was

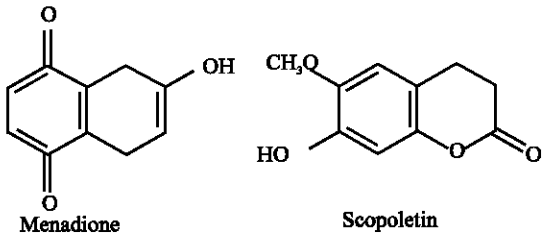


Fig. 1: Chemical structures of Scopoletin (7-hydroxy-5-methoxycoumarin; CAS 92-61-5) and Menadione (2-methyl-1,4-naphthoquinone; vitamin K_3; CAS 58-27-5)

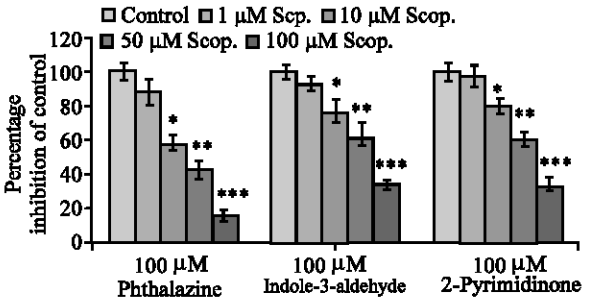


Fig. 2: Inhibition of phthalazine, indole-3-aldehyde and phenanthridine oxidation by guinea pig liver aldehyde oxidase in the presence of (1, 10, 50 and 100 μM) Scopoletin (n = 3±SD; *$p<0.05$, **$p<0.01$, ***$p<0.005$ vs. control), the control values as indicated in Table 1

therefore concluded that Scopoletin is not a substrate for guinea pig liver aldehyde oxidase or any component present in our preparation. Interestingly, Scopoletin is structurally related to menadione (Fig. 1), a known potent and selective inhibitor for aldehyde oxidase. In this study we found that Scopoletin also inhibited xanthine oxidation in our enzyme preparation which strongly indicate that the compound interact with xanthine oxidase (Table 1). However, its inhibitory effect on xanthine oxidase activity was less potent (42%) than that of aldehyde oxidase (56-88%). The inhibition of indole-3-aldehyde oxidation, catalyzed by guinea pig liver aldehyde oxidase, by Scopoletin was found to be non-competitive with an inhibitor constant (K_i) value of 57.2±3.3 μM using oxygen as electron acceptor. This result is in very close agreement with value recently shown by Lee *et al.*[2] (K_i = 52 μM) using Scopoletin and acetylcholinesterase. In fact, the last enzyme also contained Fe-group in the vicinity of the enzymatic site which is reduced from ferric ion to ferrous ion during the hydrolysis reaction and thought to be the site of inhibition by Scopoletin. This supported by the fact that inhibition of phthalazine

Table 1: Comparison of effects of Scopoletin, potent aldehyde oxidase and xanthine oxidase inhibitors on the oxidation of phthalazine, indole-3-aldehyde, 2-pyrimidinone and phenanthridine catalyzed by partially purified guinea pig liver molybdenum hydroxylase fractions

	% Inhibition of the oxidation of*				
Inhibitors (100 μM)	Phthalazine (100 μM)	Indole-3-aldehyde (100 μM)	2-Pyrimidinone (100 μM)	Phenanthridine (50 μM)	Xanthine (50 μM)
Control	0.278	0.265	0.191	0.283	0.0362
Chlorpromazine	97±2	98±3	96±3	97±2	2±1
	(0.009)**	(0.006)	(0.008)	(0.009)	(0.036)
Menadione	96±3	95±2	96±2	96±3	3±2
	(0.012)	(0.014)	(0.008)	(0.011)	(0.035)
Scopoletin	88±5	56±4	62±5	59±4	42±6
	(0.034)	(0.117)	(0.073)	(0.116)	(0.021)
Allopurinol	3±1	2±2	2±1	5±3	97±2
	(0.271)	(0.260)	(0.187)	(0.268)	(0.001)

* Results are expressed as mean percentage inhibition±SD (male/female guinea pigs, n =4),
** Result in brackets are initial rates in the present of inhibitor (μMol/min/mg protein)

Table 2: Effects of Scopoletin and menadione, a potent aldehyde oxidase inhibitor, on the oxidation of indole-3-aldehyde catalyzed by partially purified guinea pig liver molybdenum hydroxylase fractions using different electron acceptors

	% Inhibition of the oxidation of indole-3-aldehyde (50 μM)		
Electron acceptor	Chlorpromazine	Menadione	Scopoletin
Potassium ferricyanide	97±2	94±3	88±4
Cytochrome c	96±3	95±2	57±3
Oxygen	98±2	96±4	53±3

Table 3: Effects of Scopoletin, chlorpromazine and menadione on the reactive oxygen species formation during phenanthridine oxidation catalyzed by partially purified guinea pig liver molybdenum hydroxylase fractions using different electron acceptors

	% Inhibition of ROS during the oxidation of phenanthridine (50 μM)*		
ROS	Chlorpromazine (100 μM)	Menadione (100 μM)	Scopoletin (100 μM)
Superoxide anion	97±2**	98±3	83±4
Hydrogen peroxide	95±3	87±2	70±5

* The results are expressed as mean percentage inhibition±SD (male/female guinea pigs, n = 4), ** The control rates for superoxide anion and hydrogen peroxide formation are 0.048 and 0.175 μMol/min/mg protein

oxidation was more potent (88%) than other substrates (56-62%). The reason for this discrepancy is the dependence of phthalazine measurement on potassium ferricyanide ($K_3Fe(CN)_6$) reduction at 420 nm while other substrates were measured directly either following substrate disappearance or product formation directly.

Electrons egress from the enzyme can be followed using electron acceptors that interact with the enzyme at different redox centers. Potassium ferricyanide accept electrons from iron-sulfur center whereas cytochrome c is reduced by superoxide anion at FAD site. Inhibition of potassium ferricyanide was found to be more pronounced than that of cytochrome c or oxygen reduction during indole-3-aldehyde oxidation by aldehyde oxidase (Table 2). Which may indicate that Scopoletin inhibits the enzyme at other sites than molybdenum center. However, the inhibition of reactive oxygen species formation may shed more light on the specific-site of inhibition.

During aldehyde oxidase catalyzed-reactions reactive oxygen species, hydrogen peroxide and superoxide anion, are produced in substantially high amounts as reviewed recently[12]. The effect of Scopoletin on production of reactive oxygen species, hydrogen peroxide and superoxide anion, has been compared to those of chlorpromazine and menadione during the oxidation of 50 μM phenanthridine (Table 3).

The effect of Scopoletin on superoxide anion production (83%) was more pronounced than that of hydrogen peroxide formation (70%) or substrate oxidation (56-62%). Similarly, menadione inhibits the superoxide anion production more than hydrogen peroxide but more potently. In contrary, chlorpromazine has been found to be equipotent on both species. Interaction of menadione is thought to occur at FAD site[5,16], which is consistent with the ability of menadione to act as an electron acceptor of xanthine oxidase. As a result, the oxidation rates of xanthine oxidase are enhanced in the presence of menadione[16,17]. No reaction has been observed during the incubation of Scopoletin with cytochrome c or potassium ferricyanide alone which indicates that Scopoletin has no intrinsic reaction with oxidized form of iron.

Imbert and Wilson[18] have shown that Scopoletin inhibited indole-3-acetic acid (IAA) oxidase activity at high concentrations but stimulated activity at low concentration and these effect were dependent on the concentration of IAA and enzyme. In contrary, Scopoletin inhibition of aldehyde oxidase activity was found to be independent of either substrate or enzyme concentrations (Fig. 3). It should be note that IAA oxidase are prepared from sweet potato (*Ipomoea batatas*) roots while in this study the oxidase is prepared from guinea pigs. It is interesting to note that aldehyde oxidase, purified from maize seeding, although very similar to that found in

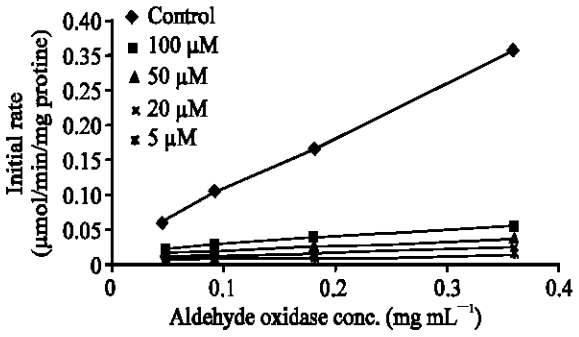
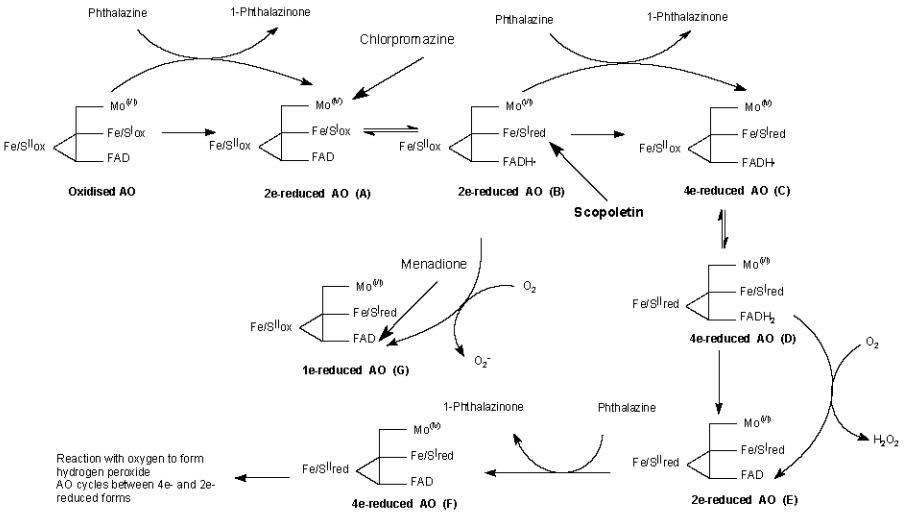


Fig. 3: Dependence of Scopoletin inhibition on phthalazine concentrations (5, 20, 50 and 100 µM) and aldehyde oxidase concentrations (0.045, 0.09, 0.18 and 0.36 mg mL^{-1}), the control values measured in absence of Scopoletin at difference substrate and enzyme concentrations (n = 3)

manmalian systems is not inhibited by menadione[19]. There are difference in NAD-binding site between the mammalian and plant aldehyde oxidase genes that may be responsible for the inability of menadione (and Scopoletin) to inhibit or even stimulate plant enzyme at the same fashion[19,20].

Scopoletin interaction with drug-metabolizing molybdenum hydroxylases has not been reported previously in literature. In this study, Scopoletin was found to be a remarkable inhibitor of guinea pig liver aldehyde oxidase with less reactivity towards xanthine oxidase. As Scopoletin inhibits superoxide anion production more effectively than hydrogen peroxide production, in conjunction with the fact that it has more inhibitory effect on potassium ferricyanide compared to that of oxygen, as electron acceptor, the site of interaction is thought to be either Fe/S-I or flavin semiquinone (FADH·). It has been shown previously that potassium ferricyanide is reduced to potassium ferrocyanide at Fe/S-I and by receiving one electron from the molybdenum center[21]. In agreement to the proposed interaction site of Scopoletin with the enzyme, initial rates of phthalazine oxidation were inhibited more potently than other substrate (Fig. 2). In fact, oxidation of phthalazine has been followed indirectly using potassium ferricyanide ($K_3Fe(CN)_6$) while the oxidation of indole-3-aldehyde and 2-pyrimidinone have been followed directly using oxygen as electron acceptor. The proposed site of Scopoletin interaction with aldehyde oxidase during substrate oxidation is shown in Scheme 1.

By analogy with the reaction between xanthine and xanthine oxidase/dehydrogenase, it can be postulated that substrate oxidation occurs at the molybdenum center reducing Mo(VI) to Mo(IV)[21]. This is thought to be the rate-limiting step in substrate oxidation[22]. It is likely that chlorpromazine inhibits the enzyme at this stage as similar effects are observed on substrate oxidation, hydrogen peroxide production and superoxide anion formation. However, in view of non-competitive nature of chlorpromazine[23] and Scopoletin inhibition, it appears that they do not bind to fully oxidized aldehyde oxidase but to a reduced forms of molybdopterin cofactor containing either Mo(IV) or Mo(V) and Fe/S $I_{red.}$ center



Scheme 1: Reduction of aldehyde oxidase by phthalazine and subsequent electron transfer to form superoxide anion and hydrogen peroxide in the presence of different inhibitors (AO: aldehyde oxidase; Scopoletin: 7-hydroxy-5-methoxycoumarin; Fe/S: iron-sulfur center)

(Scheme 1-B). This is supported by the fact that Scopoletin is a progressive inhibitor for substrate oxidation.

Rapid intra-molecular electron transfer will generate reduced Fe/S and FADH (flavin semiquinone, Scheme 1-B). Although electrons are transferred singly from Mo(IV), under steady state conditions the flavin is maintained as $FADH_2$ (flavin hydroquinone, Scheme 1-D), which favors reduction of molecular oxygen to hydrogen peroxide. During enzyme turnover, aldehyde oxidase is thought to cycle between four-electron and two-electron reduced forms thus both Fe/S groups will be reduced. Rapid equilibria will be established between FAD/FADH and $FADH/FADH_2$. The major flow of electrons will be through the four-electron reduced form containing $FADH_2$ (Scheme 1-D) whereas electron flux from FADH to form superoxide anion has been shown in this study and other studies[24,25] to account for only ~8% of total substrate turnover.

In a previous study we found that Lawsone, a natural dye of Henna, interacts with guinea pig live aldehyde oxidase (thought to be conducted at FADH)[26], in contrast Scopoletin appears to bind to Fe/SI as the effect on superoxide anion formation as well as reduction of potassium ferricyanide were more pronounced than that on hydrogen peroxide formation (Scheme 1-B).

The interaction of Scopoletin with NADH oxidation, thought to be through FAD prosthetic group rather than molybdenum site, by aldehyde oxidase and the inhibitor effect on reduction reactions droved by aldehyde oxidase will be focused on in further studies.

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