



Stilbene compounds are specific inhibitors of the superoxide anion generation catalyzed by xanthine oxidase

Noriyoshi Masuoka

Tsudaka-Fruit Juice Laboratory, Okayama Research-Park Incubation Center, 5303 Haga, Kita-ku, Okayama 701-1221, Japan

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Chemical compounds studied in this article:

Resveratrol (PubChem CID: 445154)
 Resveratrol 3-glucoside (PubChem CID: 5281718)
 Resveratrol 4'-glucoside (PubChem CID: 131751049)
 Pterostilbene (PubChem CID: 5281727)
 Piceatannol (PubChem CID: 667639)
 Rhapontigenin (PubChem CID: 5320954)
 Isorhapontigenin (PubChem CID: 5318650)
 Flavin mononucleotide sodium salt (PubChem CID: 23666409)
 Flavin adenine dinucleotide disodium salt (PubChem CID: 2734019)

ABSTRACT

The inhibitory effect of xanthine oxidase (XO) reactions with stilbene compounds, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging by stilbene compounds and superoxide anion (O_2^-) scavenging activity were examined. The inhibition of the O_2^- generation catalyzed by XO by stilbene compounds is stronger than the effect on uric acid formation. The suppression of the O_2^- generation with resveratrol was diminished by the addition of flavin adenine dinucleotide (FAD). The water-solubility and visible spectra (VIS) of the stilbene compounds in the presence of water-soluble flavin compounds indicated a π - π interaction between the stilbene compounds and the isoalloxazine in flavin compounds. These results indicate that stilbene compounds specifically bind the FAD site in XO so as to inhibit the O_2^- generation. In the case of piceatannol, it is deduced that the suppression of O_2^- generation is induced by this specific binding to the FAD site and the subsequent reduction of XO.

1. Introduction

Xanthine oxidoreductase exists in two forms, xanthine dehydrogenase (XDH) (EC 1.1.1.204) and XO (EC 1.1.3.22). XDH is converted to XO under oxidative stress. XO contains molybdenum, pterine, Fe-S and FAD as cofactors (Bay, 1975). The reaction consists of two reactions. One reaction is the oxidation of hypoxanthine to xanthine and finally to uric acid, and another is the reduction of oxygen to reactive O_2^- and hydrogen peroxide (H_2O_2). The reactions progress via a non-sequential mechanism (Fig S1 in the Supplementary material), and the reduction of oxygen is faster than the oxidation reaction of xanthine to uric acid.

The overproduction of uric acid induces hyperuricemia and gout. The overproduction of O_2^- generates reactive oxygen species (ROS) and causes oxidative stress, including lipid peroxidation (Frong, McCay,

Poyer, Keele & Misra, 1973; McCord, 1985), and constitutes a major problem for both health and the field of food manufacturing. We examined the antioxidant properties of certain natural products using XO and other reagents and indicated that the property is divided into three types of reaction (Masuoka & Kubo, 2018) (Fig S2 in the Supplementary material).

(1) Inhibition of the uric acid formation catalyzed by XO simultaneously suppresses O_2^- generation. Molecular docking indicated that the inhibitors can directly enter the xanthine-binding site of XO to inhibit XO reaction (Lin, Chen, Chen, Liang & Lin, 2002; Masuoka, Nihei & Kubo, 2006; Masuoka, Matsuda, & Kubo, 2012). (2) The suppression of O_2^- generation is associated with the conjugated en-diol structures (Fig. 1A) and is induced by the reduction of XO molecules so as to generate H_2O_2 and uric acid (Hille & Massey 1981; Masuoka, Matsuda &

E-mail address: masuokan@ms11.megaegg.ne.jp.

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Kubo, 2012). The suppressor binds to the site in the isoalloxazine ring of FAD in XO (Zhang, Zhang, Liao & Gong, 2017). (3) O₂⁻ scavenging activity is related to polyphenols having conjugated en-diol structures and alk(en)yl chains (Masuoka, Nihei, Maeta, Yamagiwa, Kubo, 2015; Masuoka & Kubo, 2016). However, inhibition of the O₂⁻ generation induced by the binding to the isoalloxazine ring of FAD in XO was not found.

In this paper, I planned to examine the antioxidant properties using XO and other reagents with stilbene compounds (Fig. 1B), as it is known that resveratrol (1) has a weak DPPH scavenging activity and ameliorates the oxidative stress induced by ROS (Ryan et al., 2010).

2. Methods

2.1. Materials

The DPPH radical, EDTA, flavin mononucleotide sodium salt (FMN), flavin adenine dinucleotide disodium salt (FAD), and other reagents were purchased from the Sigma-Aldrich Co. (Tokyo, Japan) and were of analytical grade. Stilbene compounds were obtained from Tokyo Kasei

(Tokyo, Japan).

2.2. Preparation of sample solution

Stilbene compounds were dissolved with dimethyl sulfoxide (DMSO). A 10 mM solution was prepared and examined for each.

2.3. Assay of uric acid catalyzed by XO in the presence of sample solution

The XO solution (EC 1.1.3.22, Grade IV) used for the bioassay was purchased from Sigma-Aldrich Japan. The XO reaction was performed at pH 10 in order to detect labile O₂⁻. The reaction mixture (final volume was 3.0 mL) containing 0 – 200 μM xanthine and 0.03 mL of the sample solution in 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0) at 25 °C was prepared. The reaction was started by the addition of 0.12 mL of XO (0.04 unit), and the absorbance at 293 nm was recorded for 60 s. A control experiment was carried out by replacing the sample solution with the same amount of DMSO. The reaction rate was calculated from the proportional increase of absorbance.

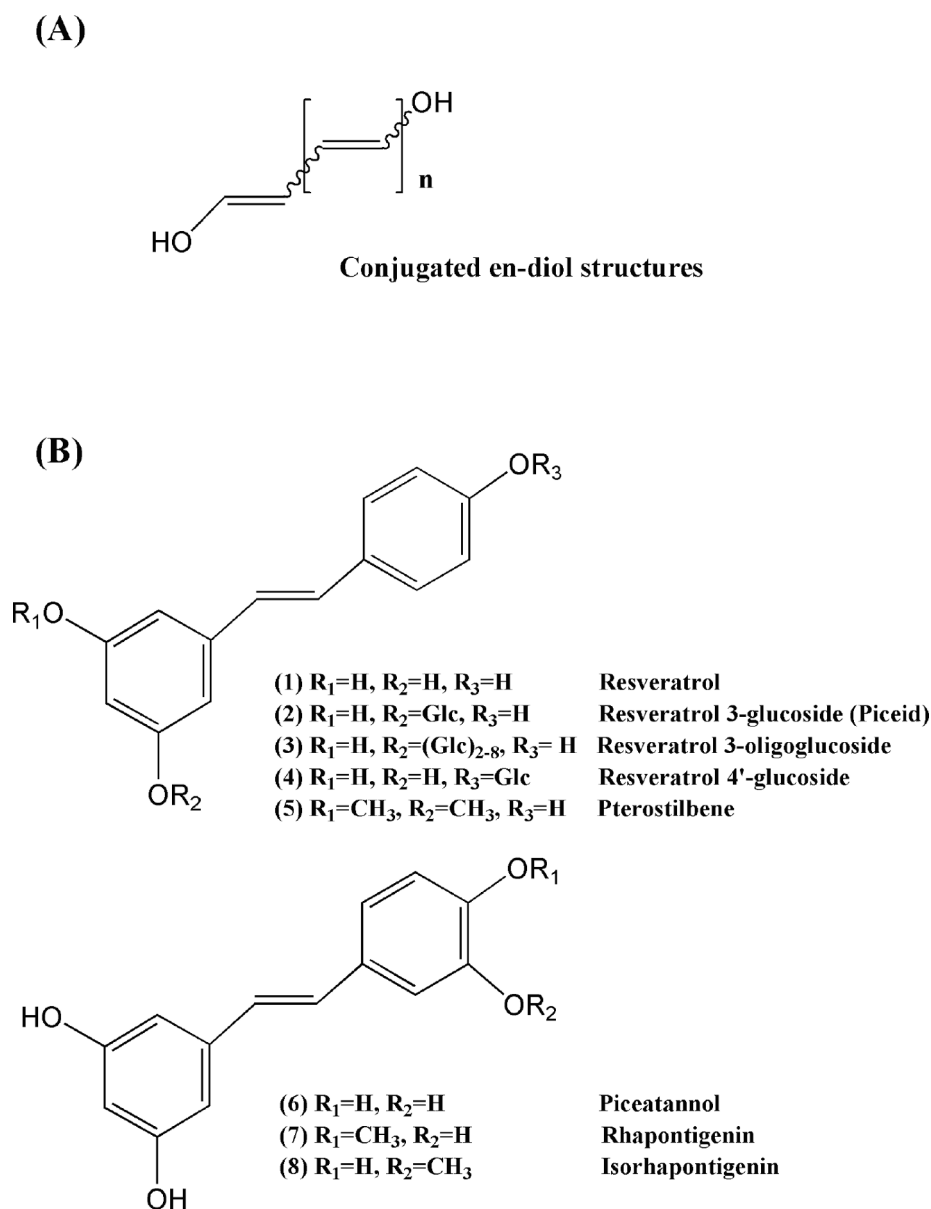


Fig. 1. Conjugated en-diol structures (A) and stilbene compounds (B).

2.4. Assay of the O₂⁻ catalyzed by XO in the presence of the sample solution

The O₂⁻ was detected by the reduction of nitroblue tetrazolium to blue formazan (560 nm) (Toda, Kumura, & Ohnishi 1991).

The reaction mixture (final volume was 3.0 mL) containing 0 – 200 μM xanthine, 0.03 mL of 0.5 % bovine serum albumin, 0.03 mL of 2.5 mM nitroblue tetrazolium and 0.06 mL of the sample solution (dissolved in DMSO) in 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0) was prepared under air atmosphere. 0.12 mL of XO (0.04 units) was added to the mixture at 25 °C to start the reaction, and the absorbance at 560 nm was recorded for 60 s. A control experiment was carried out by replacing the sample solution with the same amount of DMSO. The reaction rate was calculated from the proportional increase of the absorbance.

2.5. Scavenging of DPPH radicals and the initial scavenging rates

One mL of 100 mM acetate buffer (pH 5.5), 1.87 mL of ethanol and 0.1 mL of an ethanolic solution of 3 mM DPPH were put into a test tube (Blois, 1958). Then 0.03 mL of the sample solution was added to the tube and incubated at 25 °C for 20 min. The absorbance at 517 nm (DPPH, ε = 8.32 × 10³) was recorded. As a control, 0.03 mL of DMSO was added to the tube. From the decrease in the absorbance during a period of 20 min, the scavenging activity was calculated and expressed as the DPPH molecules scavenged per sample molecule. The initial rate of the scavenging activity was calculated from the decrease in the absorbance after the addition of sample solution (Masuoka, Isobe & Kubo, 2006).

2.6. Scavenging of the O₂⁻ generated by the PMS-NADH system

The superoxide anion was generated nonenzymatically with a PMS-NADH system (Nishikimi, Rao & Yagi, 1972). The reaction mixture (the final volume was 3.0 mL) consisted of 2.82 mL of 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0), 0.03 mL 0.5 % bovine serum albumin, 0.03 mL of 2.5 mM nitroblue tetrazolium, 0.06 mL sample solution and 0.03 mL of 7.8 mM NADH. The mixture was maintained at 25 °C and the reaction carried out. The reaction was started by the addition of 0.03 mL of 155 μM PMS, and the absorbance at 560 nm was recorded for 60 s. 0.06 mL of DMSO was used as the control. The reaction rate was calculated from the proportional increase absorbance, and the scavenging activity was calculated using the following equation.

$$\text{Scavenging activity(\%)} = [1 - (\text{rate of sample})/(\text{rate of control})] \times 100$$

2.7. Water-solubility of the stilbene compounds in the presence of the flavin compounds

One mL of 0, 5, 10, 20, and 25 mM FAD (or FMN) aqueous solution was added to powders (ca 10 mg) of a stilbene compound. Each suspension was sonicated for 30 min and allowed to stand for 1 h. After centrifugation, the supernatant was separated as a saturated solution. A portion of the supernatant was diluted with 0.1 M citrate buffer (pH 4.0) to a less than 0.1 mM flavin concentration. The absorbance at the maximum absorption wavelength of the stilbene compound was recorded, and the content was calculated. For example, resveratrol content was calculated using the increase in the absorbance at 317 nm (ε = 29,000).

2.8. VIS spectra of the stilbene compounds in 10 mM FAD or FMN solution

Each stilbene compound was added to an aqueous solution of 10 mM FAD or FMN. The VIS-spectra of the solution were recorded using a cuvette having a light-path length of 0.1 mm.

2.9. Assay and data analysis

Each assay was performed more than three times in separate experiments, and the analysis was performed with Sigma plot 2001 (SPSS Inc., Chicago, IL). The inhibition mode and kinetic parameters were analyzed with Enzyme Kinetics Module 1.1 (SPSS Inc.) equipped with Sigma Plot 2001.

3. Results

3.1. Effect of stilbene compounds on the uric acid formation catalyzed by XO

The uric acid formation catalyzed by XO was examined in the presence of stilbene compounds (1–8). The absorbance at 293 nm indicated that the measurement of uric acid formation was only possible for concentrations of less than 0.1 mM of the stilbene compounds due to an overlapping of the absorption data for uric acid and the stilbene compound. The results are summarized in Table 1. The inhibition kinetics of uric acid formation by xanthine with stilbene compounds (1, 3, 6–8) was analyzed as competitive inhibition. Rhapontigenin (7) was the strongest inhibitor among them.

3.2. Effect of stilbene compounds on the suppression of the O₂⁻ generation catalyzed by XO

When the xanthine concentration in the reaction was changed to 0 – 200 μM, the O₂⁻ generation catalyzed by XO was suppressed by the stilbene compounds. The results are summarized in Table 2. The suppression of O₂⁻ generation by resveratrol and related compounds (1–5, 7, 8) was analyzed as competitive inhibition. The kinetics of O₂⁻ generation by piceatannol (6) was analyzed as a mixed type using caffeic acid derivatives (Masuoka & Kubo, 2016). Piceatannol is the strongest suppressor of O₂⁻ generation among them.

When inhibitors of uric acid formation bound to the xanthine binding site in XO, the O₂⁻ generation was suppressed since the uric acid formation was slower than O₂⁻ generation. Compared with this, the stilbene compounds inhibited O₂⁻ generation more potently than the uric acid formation catalyzed by XO (Table 1 and 2). It is suggested that the stilbene compounds bind to the FAD site in XO and inhibit O₂⁻ generation, after which the uric acid formation is suppressed.

3.3. The inhibition by resveratrol of the O₂⁻ generation catalyzed by XO in the presence of FAD

The inhibition by resveratrol of the O₂⁻ generated by XO was examined in the presence of FAD. The addition of FAD did not affect the O₂⁻ generation rate in the XO reaction and moderately suppressed the inhibition of O₂⁻ generation by resveratrol. The IC₅₀ values of resveratrol and resveratrol-FAD (1:4) mixture were 21 ± 3 and 70 ± 4 μM, respectively. It is suggested that resveratrol binds to the FAD site in XO and FAD molecules in the reaction mixture.

Table 1
Inhibition of uric acid formation using xanthine oxidase by stilbene compounds.

Compound	Ki (μM)	IC ₅₀ (μM)*
Resveratrol (1)	7.7 ± 0.7	34 ± 4
Resveratrol-3-glucoside (2)	–	>50
Resveratrol-4'-glucoside(3)	7.0 ± 0.8	25 ± 4
Resveratrol-3-oligoglucoside(4)	–	>50
Pterostilbene (5)	–	>50
Piceatannol (6)	6.7 ± 1.6	15 ± 5
Rhapontigenin (7)	3.5 ± 0.2	7.9 ± 0.5
Isorhapontigenin (8)	96 ± 2	161 ± 11**

* IC₅₀ values at 200 μM xanthine were measured.

** The value was calculated from the kinetic parameters.

Table 2
Inhibition O₂⁻ generation using xanthine oxidase by stilbene compounds.

Compound	K _i (μM)	K _i ' (μM)	IC ₅₀ (μM)*
Resveratrol (1)	7.1 ± 1.0	–	21 ± 3
Resveratrol-3-glucoside (2)	19 ± 2	–	49 ± 1
Resveratrol-4'-glucoside(3)	5.0 ± 0.1	–	11 ± 2
Resveratrol-3-oligoglucoside(4)**	121 ± 19**	–	323 ± 20**
Prostilbene (5)	43 ± 5	–	124 ± 16
Piceatannol (6)	2.2 ± 0.1	21.1 ± 1.0	4.5 ± 0.4
Rhapontigenin (7)	2.4 ± 0.2	–	6.0 ± 1.0
Isorhapontigenin (8)	36 ± 3	–	99 ± 8

* IC₅₀ values were measured at 200 μM xanthine. ** This compound was a mixture of resveratrol-3-(Glc)₂₋₈ and the average molecular weight was 807.

To further investigate this phenomenon, the water-solubility and UV–VIS spectra of the stilbene compounds were examined in the presence of water-soluble flavin compounds.

3.4. DPPH scavenging activity and the initial scavenging rate of the stilbene compounds

The activity of DPPH scavenging is related to the capacity for electron-donation and is useful for evaluating the reducing capacity of XO molecules (Masuoka, Matsuda & Kubo, 2012). The scavenging results are summarized in Table 3. Resveratrol and the derivatives (1–5) displayed a weak scavenging activity similar to the alk(en)yl phenols. As the scavenging activity of piceatannol and its derivatives (6 – 8) is considerable, the initial scavenging rates were examined. As the initial scavenging rate of piceatannol is higher than the rates of rhapontigenin and isorhapontigenin, it is deduced that piceatannol is able to reduce XO and suppresses O₂⁻ generation. This is consistent with the structure of piceatannol, which has a catechol (conjugated en-diol) structure.

3.5. O₂⁻ scavenging activity of the stilbene compounds using the PMS-NADH system.

The scavenging reaction is a stoichiometric radical reaction, and the activity of stilbene compounds is indicated in Fig. 2. Piceatannol is a potent O₂⁻ scavenger, and the IC₅₀ value is estimated to be 9.0 ± 2.0 μM. Compared to the IC₅₀ values of other phenolic compounds having conjugated en-diol structures, caffeic acid and gallic acid were 51 and 29 μM, respectively (Masuoka, Nihei, & Kubo, 2006; Masuoka & Kubo, 2016). This finding suggests that piceatannol radical has greater stability than the phenolic radicals because of the length of its conjugated en-diol structure.

3.6. Water-solubility of the stilbene compounds in the presence of FAD or FMN

The water-solubility of resveratrol was low (0.19 mM). When resveratrol was added to the aqueous solution of water-soluble flavin

Table 3
DPPH scavenging activity and the initial scavenging rate of stilbene compounds.

Compound	DPPH activity*	Initial rate**
Resveratrol (1)	0.72 ± 0.27	ND
Resveratrol-3-glucoside (2)	0.59 ± 0.15	ND
Resveratrol-3-oligoglucoside (3)	0.59 ± 0.15	ND
Resveratrol-4'-glucoside(4)	0.55 ± 0.17	ND
Pterostilbene (5)	0.74 ± 0.10	ND
Piceatannol (6)	2.45 ± 0.13	3.75
Rhapontigenin (7)	2.21 ± 0.22	1.27
Isorhapontigenin (8)	2.10 ± 0.21	1.77

*DPPH activity was indicated as DPPH molecules / a molecule of stilbene compound. The DPPH activity of lauryl gallate was 7.32 ± 0.04. **The initial scavenging rate was indicated using μmol / L / s.

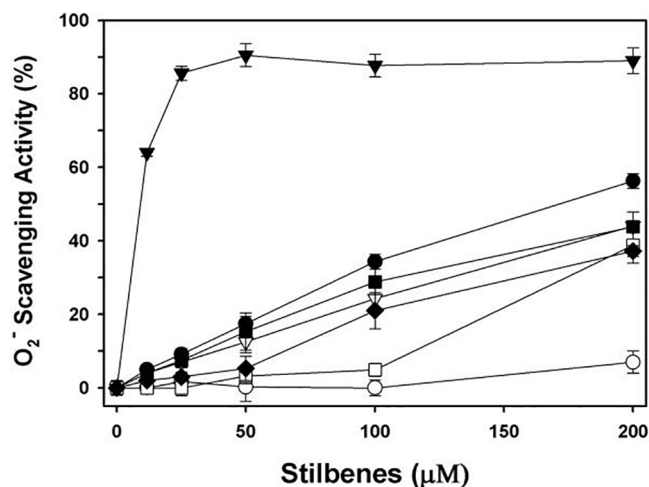


Fig. 2. Suppression of the O₂⁻ generation by the stilbene compounds using the PMS-NADH system. Stilbene compounds; resveratrol (●), piceid (○), resveratrol-4'-glucoside (◆), pterostilbene (□), piceatannol (▼), rhapontigenin (■) and isorhapontigenin (▽).

compounds (FAD or FMN), the solubility increased. The increase is in proportion to the concentration of the flavin compounds, as indicated in Fig. 3A. The water-solubility of the other stilbene compounds added to aqueous FMN solution is shown in Fig. 3B. These results indicate that stilbene compounds interact with flavin compounds and suggest that the interaction augments the water-solubility of the stilbene compounds.

3.7. VIS spectra of the stilbene compounds in 10 mM FAD or FMN solution

When each of the stilbene compounds was dissolved in aqueous FAD or FMN solution, the color of the solution changed from yellow to orange-red. To examine this change, the VIS spectrum of the solution was recorded using a cuvette having a light-path length of 0.1 mm. The spectrum of 2.5 mM resveratrol solution in 10 mM FAD or FMN indicated the same spectrum, and the λ_{max} (ε) was 336 nm (10,100), 376 nm (8,070), 448 nm (9,920) and 510 nm (7,300). That of 6.67 mM piceid in 10 mM FMN solution indicated the λ_{max} (ε) to be 328 nm (21,100), 380 nm (6,900), 449 nm (8,730) and 510 nm (1,700). That of 10 mM piceatannol in 10 mM FMN solution indicated the λ_{max} (ε) to be 331 nm (27,800), 377 nm (6,490), 449 nm (8,330) and 510 nm (2,020). (The spectrum of 6.67 mM piceid in 10 mM FMN solution is shown in Fig S3 in the Supplementary material.) These findings indicate that stilbene compounds interact with the isoalloxazine ring in the flavin compounds, and the color change is due to the absorbance at 510 nm.

4. Discussion

Stilbene compounds more potently inhibited the O₂⁻ generation than the uric acid formation catalyzed by XO (Table 1 & 2). The low DPPH scavenging activity indicated that the stilbene compounds except for piceatannol were unable to reduce XO molecules (Table 3). When the O₂⁻ generation reaction catalyzed by XO with resveratrol was examined in the presence of FAD, the inhibition of O₂⁻ generation by resveratrol was moderately decreased by FAD. These suggested that stilbene compounds specifically bind to the FAD site in XO to inhibit O₂⁻ generation and then the uric acid formation is eventually suppressed. To further examine the binding between the stilbene and flavin compounds, the water-solubility of stilbene compounds for aqueous FAD or FMN solution was examined. The solubility increased in proportion to the concentration of FAD or FMN (Fig. 3A and 3B). This suggests that stilbene compounds interact with the flavin compounds so as to increase their solubility. When stilbene compounds were added to flavin compounds in

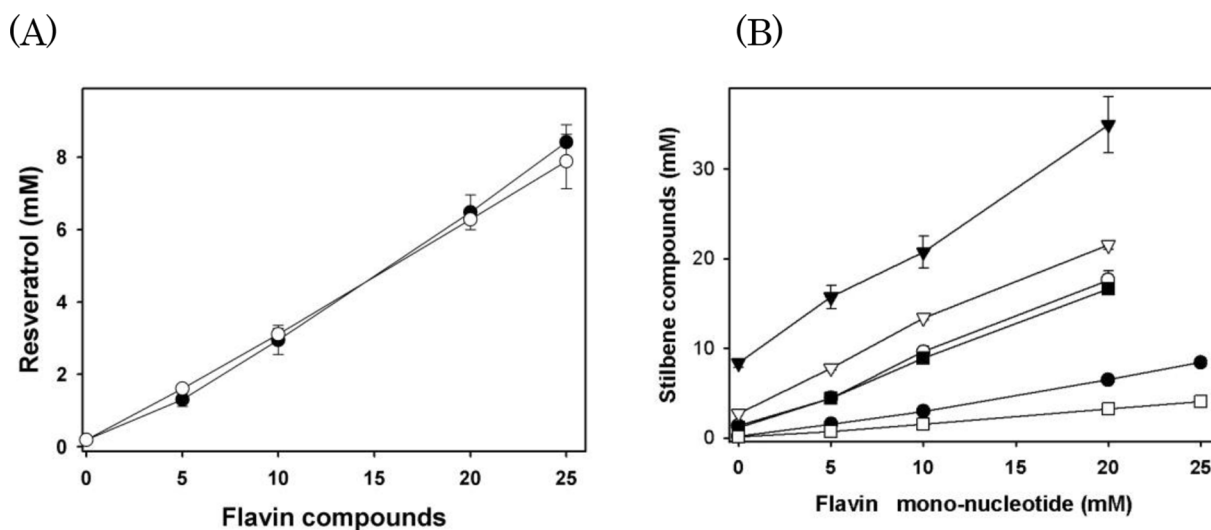


Fig. 3. Water-solubility of stilbene compounds in the presence of flavin compounds. (A) Water-solubility of resveratrol with FAD or FMN solution. FAD (○) and FMN (●). (B) Water-solubility of stilbene compounds with FMN solution. Stilbene compounds; resveratrol (●), piceid (○), pterostilbene (□), piceatannol (▼), isorhapontigenin (▽) and rhapontigenin (■).

aqueous solution, the color changed from yellow to orange-red. The VIS spectra indicated a π - π interaction between the stilbene compounds and the isoalloxazine ring in the flavin compounds. As these findings indicate that the binding of stilbene compounds to the FAD site in XO suppresses the O_2^- generation rather than uric acid formation, it is confirmed that the O_2^- generation induced by the increase of XO under oxidative stress is suppressed by the stilbene compounds. This may explain why resveratrol attenuates the oxidative stress induced by increase of XO activity, which is observed with exercise (Ryan et al., 2010) or nicotine administration (Hamza & El-Shenawy, 2017).

The suppression of O_2^- generation with piceatannol (6) is more potential than O_2^- generation with rhapontigenin (7), though inhibition of uric acid formation with rhapontigenin is stronger than that with piceatannol (Table 1 & 2). As piceatannol can reduce XO molecules because of the conjugated en-diol structure, the suppression of O_2^- generation was induced by the reduction of XO in addition to the binding to the FAD site.

Furthermore, as piceatannol has potent O_2^- scavenging activity (Fig. 2), it is suggested that piceatannol suppresses the O_2^- generation induced by antimycin A more than it is suppressed by resveratrol and thereby helps prevent apoptosis (Hosoda et al., 2021).

5. Conclusion

Stilbene compounds bind to the FAD site in XO and inhibit the O_2^- generation rather than uric acid formation. It is deduced that the compounds attenuate the oxidative stress. In the case of piceatannol, it suppresses O_2^- generation by the reduction of XO in addition to the binding to the FAD site and has a strong O_2^- scavenging activity.

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Author contribution

N. Masuoka carried out investigation, analysis and writing of this article.

CRedit authorship contribution statement

Noriyoshi Masuoka: Investigation, analysis and writing of this

article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2021.100146>.

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