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Scopoletin and Morin Inhibit Lactate Dehydrogenase Enzyme Activity, Which Is Critical for Cancer Metabolism

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Abstract

Background: Lactate dehydrogenase (LDH) is a tetrameric enzyme that catalyzes the interconversion of pyruvate to L-lactate. The importance of this enzyme is because LDH isoenzymes are involved in cancer, heart, and liver diseases. Inhibition of this enzyme can help prevent and treat different diseases. Morin is a flavonoid found in the *Moraceae* family and scopoletin is a coumarin found in *Scopolia* genus.

Objectives: The aim of this study was to determine the effect of morin and scopoletin as two natural products on the activity and structure of lactate dehydrogenase enzyme.

Methods: Morin and scopoletin were examined for inhibition of the activity of LDH in 100 mM sodium phosphate buffer pH 7.5, at room temperature using UV-V spectrophotometry. Fluorescence spectroscopy was used to characterize protein structural changes in the presence of morin and scopoletin.

Results: K_m and V_{max} of LDH for pyruvate were 11.69 mM and 1.258 mM/min, respectively. The kinetic results showed that morin and scopoletin are LDH inhibitors. The Ki values of morin and scopoletin were determined as 1.78 μM and 0.8 μM, respectively, using a secondary plot. Fluorescence intensity quenching and red shift of the maximum wavelength of emission in a concentration-dependent manner showed that morin and scopoletin bind to LDH and affect its structure.

Conclusions: The results suggest that morin and scopoletin bind to LDH, influence its conformation and inhibit its activity. Scopoletin showed more effective inhibition of LDH activity and it can be a promising candidate in the field of tumor metabolism inhibitors.

Keywords: Lactate Dehydrogenase, Morin, Scopoletin, Spectrophotometry, Enzyme inhibitors

1. Background

Lactate dehydrogenase (LDH, EC 1.1.1.27) is a key enzyme in anaerobic respiration that catalyzes the reversible conversion of pyruvate to lactate in the presence of NADH. Two major forms of LDH found in a wide range of organisms are the A form (A4-LDH), found predominantly in anaerobic tissues, such as skeletal muscle and liver, and the H form (B4-LDH), which predominates mainly aerobic tissues, such as the heart (1, 2). A third type has also been identified, the X form, which appears to be restricted to the testes (3). The LDH-A and LDH-B subunits are similar in size and share 75% sequence identity yet have different catalytic properties; the A subunit preferentially converts pyruvate to lactate, while for the B form the opposite is true. They organize four distinct enzyme classes with tetrameric structure; A4, A3B, A2B2, B3A, and B4 that are different isoenzymes of LDH (4). Furthermore, LDH is a biomarker of different diseases, thus this enzyme is an interesting enzyme for scientists.

Since metabolism is impaired in cancer cells, these cells are dependent on anaerobic metabolism to survive. Lactate dehydrogenase inhibition could be the target of different cancer therapy studies. Inhibition of LDH devastate glucose metabolism in cancer cells without affecting the energetic balance of normal cells. Inactivation of LDH enzyme in the presence of different natural and synthetic components has been studied. For example, Galloflavin is a synthetic compound that inhibits both A and B isoforms of LDH and hinders the proliferation of tumor cells by blocking glycolysis. The inhibition is a mixed type for pyruvate and lactate (5). FX11 [3-dihydroxy-6-methyl-7-(phenylmethyl)-4-propylnaphthalene-1-carboxylic acid] is another inhibitor of LDH that used for treatment of pancreatic cancer in vivo and in vitro (2). Genistein is another LDH inhibitor that inhibits the activity of enzyme due to competition with NADH for the enzyme binding (6). Modification of LDH enzyme can induce conformational

and activity changes. When cysteine residues of LDH react with p_chloromercuribenzoate (pCMB), the enzyme partially unfolds (7). The effect of o-phthalaldehyde (opta) on the structure and activity of Lactate dehydrogenase was investigated. Opta not only inhibited this enzyme but also changed the structure of lactate dehydrogenase and induced partial unfolding (8).

Phenolic compounds, like flavonoids and coumarins, are a wide class of plant compounds that present in almost all foods of plant origin. These compounds possess several biological properties and cause protection against different diseases, especially cardiovascular diseases and different cancers. Various researches demonstrated that their pharmacological activities depend on their antioxidant properties (9). Morin (2', 3, 4', 5, 7-pentahydroxyflavone), a natural flavonoid, can be found in almond, fig, other *Moraceae* family and also some herbal medicines. Recently cell protecting effect of morin against hydrogen peroxide-induced oxidative stress was reported. For example, in irradiated cells that produce ROS, ROS scavenging might be the reason of morin cytoprotective effects against the damage caused by gamma rays (10). Morin has been introduced as an inhibitor of xanthine oxidase, urate oxidase, amino peptidase N, phosphatidil inositol phosphate kinase, and malic enzymes (11-14). Scopoletin (6-methoxy-7-hydroxy coumarin) is one of the important members of the coumarin group. It has shown that scopoletin has anti-bacterial and antifungal properties (15, 16). Scopoletin could have an important role in the management of many symptoms and diseases, such as convulsions, inflammation, rheumatic pains, and leprosy (17). Scopoletin has shown strong inhibition of acetylcholinesterase and thus it is a potential candidate for Alzheimer's disease treatment (18). Furthermore, scopoletin has been introduced as inhibitor of β-glucuronidase, peroxidase, acetylcholinesterase, and mono amine oxidase (19-22).

2. Objectives

Because of the clinical benefits of morin and scopoletin as well as the importance of lactate dehydrogenase enzyme in cancer treatment, lactate dehydrogenase activity and its structural changes was investigated in the presence of morin and scopoletin in the current study.

3. Methods

In this experimental research, rabbit muscle LDH (L-1254), sodium pyruvate (P2256), NADH (N8129), morin (M4008), and scopoletin (S2500) were obtained from Sigma-Aldrich. Ethanol and phosphate buffer were purchased from Merck. All solutions were prepared with distilled water.

3.1. LDH Activity and Inhibition Assay (Kinetic Studies)

The LDH samples (0.75 u/mL) in 100 mM sodium phosphate buffer (pH 7.5) were freshly prepared and assayed by spectrophotometer, according to the sigma protocol (23). One unit will reduce 1.0 μmole of pyruvate to L-lactate per minute. The absorbance change produced by 0.13 mM NADH oxidation was recorded at 340 nm. The activity was determined at 0.3, 0.6, 0.9, 1.3, 1.9, 2.3, 2.6, 2.9, 3.3, 5, 6.6, and 8 mM pyruvate. The LDH activity was investigated at 1.9, 3.3, 6.7, and 8.2 μM of morin and at 0.3, 0.6, 1.3, 1.9, and 2.6 μM scopoletin. Morin and scopoletin were initially dissolved in ethanol. Kinetic parameters, K_m, and V_{max} were obtained from line weaver-burk plot and the secondary plot was used for calculating K_i values. The ultraviolet spectrum was recorded at room temperature on a sp-1200 uv spectrophotometer and 1 cm quartz cuvette.

3.2. Fluorescence Spectroscopy

Fluorescence spectroscopy studies were performed using Cary-Eclipse spectrofluorometer (Varian) measuring the fluorescence intensity of LDH (6.6×10^{-3} μM) in the absence and presence of different concentrations of morin and scopoletin. The fluorescence spectra were recorded in a cuvette of 1 cm light path at room temperature. The excitation wavelength was 280 nm and emission wavelength was in the range of 300 to 400 nm. The LDH was titrated by the stock solution of inhibitors separately, so the LDH/morin molar ratios were 1: 0, 1: 100, 1: 250, 1: 500, 1: 750, 1: 1500, 1: 2000, 1: 2500, and 1: 3000 and the LDH/scopoletin molar ratios were 1: 0, 1: 50, 1: 100, 1: 150, 1: 200, and 1: 250.

4. Results

Essentially increasing of substrate concentration increases the activity of enzyme. Line weaver-Burk plot can be used to determine K_m and V_{max} values of the LDH enzyme (Figure 1). In the current experimental condition, the K_m value was 11.69 mM and V_{max} value was 1.258 mM/min for pyruvate. In the presence of both morin and scopoletin, the LDH activity was reduced. The kinetic analysis of LDH inhibition were identified from line weaver-burk plot. K_m and V_{max} values changed in the presence of different concentrations of each inhibitor. The secondary plot of slope versus [I] was used according to Equation 1 to determine K_i.

$$Slope = \frac{K_m}{V_{max}} \times \left(1 + \frac{[I]}{K_i}\right) \quad (1)$$

Where, slope is (K_m/V_{max}) of Lineweaver-Burk plot and [I] is inhibitor concentration. The K_i value of morin was obtained as 1.78 μM and the K_i value of scopoletin was 0.8 μM (Figure 2).

Relative activity of the LDH enzyme was calculated in the presence of different concentrations of scopoletin and

morin. As shown in Figure 3, scopoletin inhibited LDH enzyme at lower concentrations compared to morin. IC50 values of LDH were found as 7.2 μM for morin and 1.7 μM for scopoletin. Therefore, scopoletin is a more potent LDH inhibitor than morin.

The intrinsic fluorescence has been widely used to study the interaction between ligands and proteins. Conformational changes of proteins alter microenvironment of aromatic residues like Trp, Tyr, and Phe. Trp is the major fluorophore of proteins intrinsic fluorescence and LDH has several Trp residues. The fluorescence spectrum of the native LDH shows a maximum at 342 nm. As shown in Figure 4, increasing of the concentration of morin, decreased the LDH intrinsic fluorescence emission, consistently. The maximum emission wavelength was shifted in the presence of morin, so that 9 nm red shift was observed in the presence of 19.5 μM morin (Figure 4). This quenching and red shift indicated that morin could bind to and induce conformational changes in the LDH. The LDH quenching mechanism was investigated using Stern-Volmer Equation 2:

$$\frac{F_0}{F} = 1 + K_{SV}\,[Q] \\ = 1 + k_q \tau\,[Q] \quad (2)$$

Where F_0 and F are the fluorescence intensities in the absence and presence of quencher (Inhibitor), respectively, [Q] is the quencher concentration, and K_{SV} is the Stern-Volmer quenching constant. k_q is the biomolecular quenching rate constant and τ_0 is the average lifetime of the Trp as fluorophore in the absence of quencher.

According to Equation 2, the K_{sv} value was calculated by linear regression of a plot of F_0/F versus [Q] (24). K_{SV} was calculated from slope of Stern-Volmer plot of LDH interaction with morin 0.948 $\times$ 10^5 M^{-1} (Figure 5).

K (binding constant) and n (number of binding sites) was calculated using Equation 3 (25).

$$log\left[\frac{(F_0 - F)}{F}\right] = logK + nlog\,[Q] \quad (3)$$

The Equation 3 plot of log (F_0-F)/F versus log [Morin] shows a slope of n and the vertical-intercept of logK. As shown in Figure 6, K and n obtained from plot of morin binding to LDH were 1.568 $\times$ 10^4 M^{-1} and 0.84, respectively.

As seen in Figure 7, LDH intrinsic fluorescence emission was investigated in the presence of scopoletin 0 to 1.62 μM. The fluorescence spectra of LDH were quenched by scopoletin and about 7 nm of red shift in the wavelength of maximum fluorescence emission was observed. K_{SV} was calculated from slope of Stern-Volmer plot for scopoletin 2.164 $\times$ 10^5 M^{-1} (Figure 8). According to Figure 9, binding constant and number of binding sites of scopoletin on LDH were 642.6 M^{-1} and 0.57, respectively (26).

The fluorescence intensity of LDH decreased with increase in both inhibitors. By quenching of LDH with an increasing LDH/inhibitor ratio, the inhibitor interaction with LDH changed the microenvironment of Trp residues and tertiary structure of the enzyme.

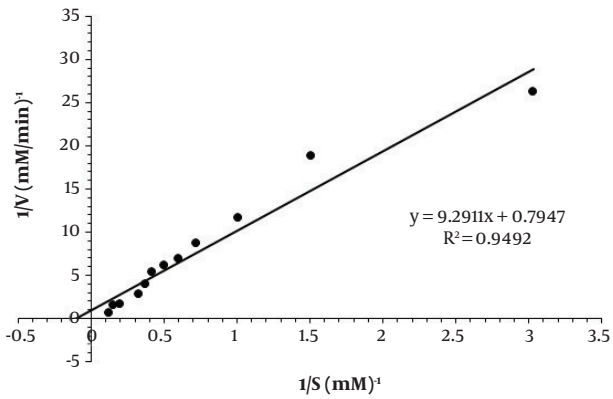

Figure 1. Lineweaver-Burk plot of LDH in the presence of different concentrations of pyruvate (0.3 to 8 mM). The enzymatic reaction was carried out in the presence of 0.075 units of LDH and 0.13 mM NADH in sodium phosphate buffer (100 mM) at pH 7.5 and room temperature.

5. Discussion

LDH interacts with morin and scopoletin and the effect of these two phenolic compounds on the activity and structure of LDH was investigated. Kinetic results indicate that both of these natural components inhibited LDH enzyme activity. Morin is structurally similar to some known LDH inhibitors, such as galoflavin and genistein. Comparison of Ki showed that inhibition potency of morin (Ki = 7.2 μM) is comparable to galloflavin (Ki = 5.46 μM) (5) and LDH was even better inhibited by scopoletin (Ki = 1.7 μM). Furthermore, scopoletin showed smaller IC50 value compared to morin and genistein (6) thus it can be introduced as a potent inhibitor of LDH.

Intrinsic fluorescence spectroscopy results showed that morin and scopoletin have the ability to bind to LDH and alter its conformation. Interaction of morin and scopoletin with LDH were of static type so inhibitors bind to LDH and approximately one inhibitor interacted with each LDH molecule. The current study observed a slight red shift in fluorescence intensity. In the presence of morin, maximum emission spectrum shifted to 351 nm (9 nm red shift) and in the presence of scopoletin shifted to 349 nm (7 nm red shift). According to the current results inhibition of LDH by morin and scopoletin is accompanied by partial unfolding. Although inactivation of LDH is often accompanied with conformational changes of this enzyme,

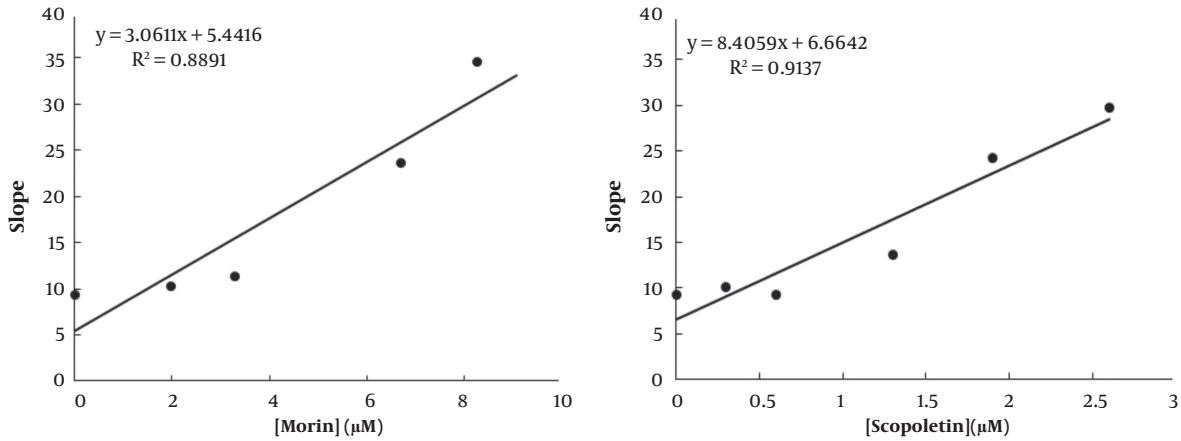

Figure 2. Secondary plots of slope versus inhibitor concentrations to calculate the inhibition constant (Ki)

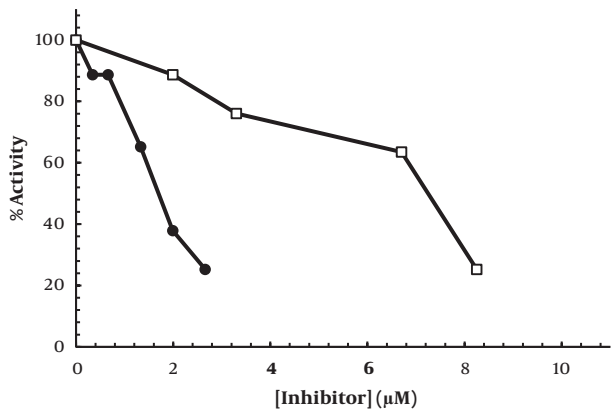

Figure 3. Effect of different concentrations of morin (□) and scopoletin (●) on the activity of LDH in 100 mM phosphate buffer, pH 7.5 in the presence of 0.3 mM pyruvate and 0.13 mM NADH

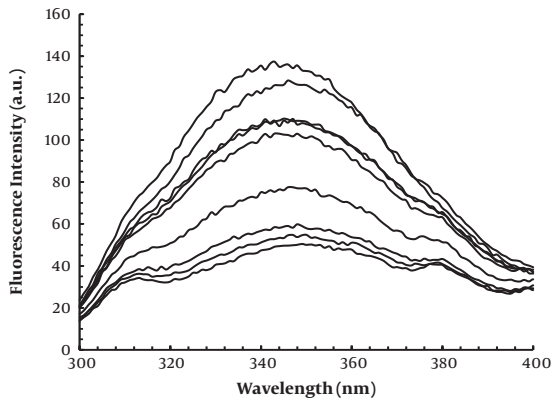

Figure 4. Intrinsic fluorescence emission spectra of LDH in absence and presence of various concentration of morin at room temperature and excitation wavelength of 280 nm. The arrow shows the concentration increase of morin and molar ratios of LDH/Morin were 1: 0, 1: 100, 1: 250, 1: 500, 1: 750, 1: 1500, 1: 2000, 1: 2500 and 1: 3000, respectively.

the amount of inhibition is not always directly subjected to structural changes.

The limitation of this study was the use of ethanol as a solvent for morin and scopoletin because they are poorly soluble in water. In a separate experiment, the highest ethanol concentration (that was used) did not affect LDH activity, hence, it could be said that the observed changes were due to ligands not the ethanol present in the sample. In this study, morin changed the structure of the enzyme more than scopoletin, while scopoletin showed more effective inhibition. It is possible that morin and scopoletin mediated impairment of activity of lactate dehydrogenase and contributed to their anticancer properties. In conclusion, scopoletin might be a promising candidate in the field of tumor metabolism inhibitors, and deserves a more exhaustive evaluation as a potential anticancer agent.

Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

Footnotes

Conflict of Interests: None declared.
Funding/Support: None declared.

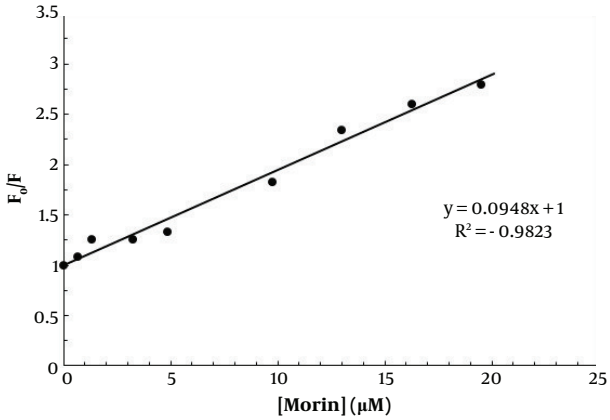

Figure 5. Stern-Volmer plot of the fluorescence quenching of LDH by morin at excitation wavelength of 280 nm

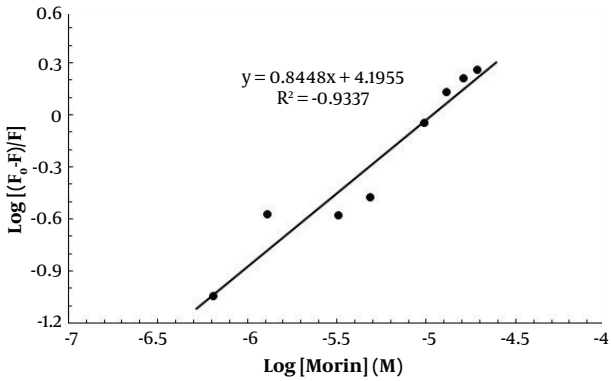

Figure 6. The plot of log $(F_0-F)/F$ versus log [Morin] for LDH in 100 mM phosphate buffer at pH 7.5

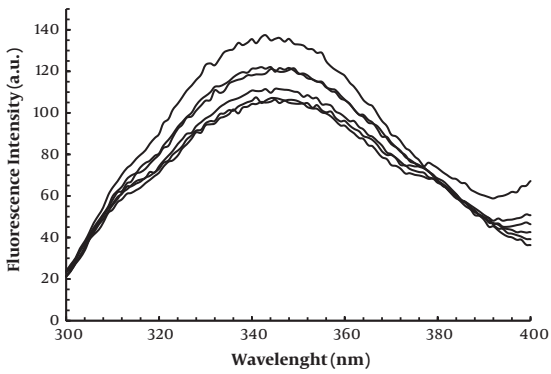

Figure 7. Intrinsic fluorescence emission spectra of LDH in the absence and presence of various concentrations of scopoletin at room temperature and excitation wavelength of 280 nm. The arrow shows the concentration increase of scopoletin. The LDH/scopoletin molar ratios were 0, 1: 50, 1: 100, 1: 150, 1: 200, 1: 250, respectively.

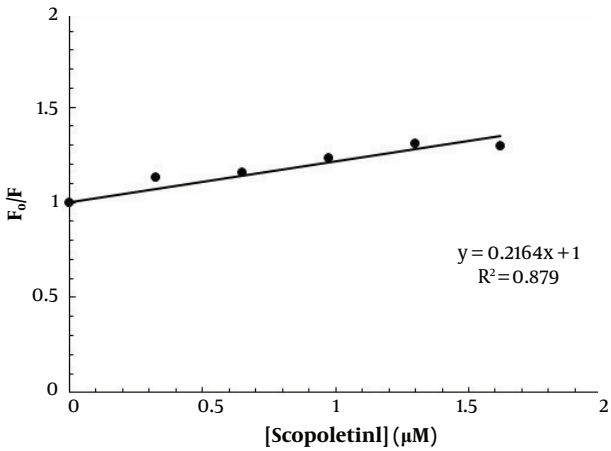

Figure 8. Stern-Volmer plot of the fluorescence quenching of LDH by scopoletin at excitation wavelength of 280 nm

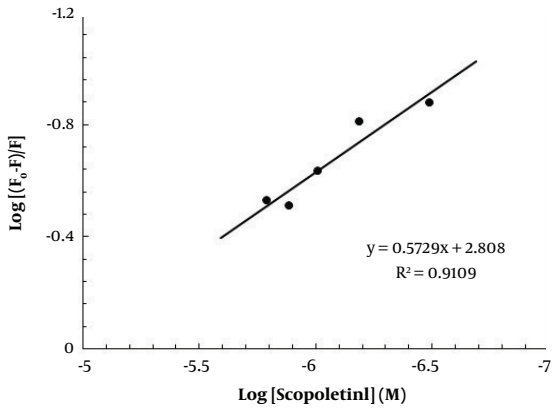

Figure 9. The plot of log $(F_0-F)/F$ versus log [Scopoletin] for LDH in 100 mM phosphate buffer at pH 7.5

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