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Inhibition of liver alanine aminotransferase and aspartate aminotransferase by hesperidin and its aglycone hesperetin: An *in vitro* and *in silico* study

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ABSTRACT

Aims: This study aimed to investigate the inhibitory effects of two natural flavonoids, hesperetin (HT) and hesperidin (HD), on two gluconeogenesis enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and their possible mechanisms of action.
Main methods: Rat liver incubated with different concentrations of HT and HD was used to measure enzyme activities spectrophotometrically, based on monitoring the oxidation of NADH to NAD^+ at 340 nm. Molecular docking simulation was also applied to reveal the molecular mechanism of the inhibition caused by HT and HD.
Key findings: Both flavonoids demonstrated inhibitory effects against the enzyme activities, with IC_{50} values of 153.9 and 68.88 μM for HT-ALT and HD-ALT treatment respectively. Likewise, the IC_{50} values of 85.29 μM for HT-AST and 110.3 μM for HD-AST were obtained from spectrophotometric results.
Conclusion: The docking simulation revealed that HT and HD block the enzyme entrance channel and prevent the substrates from accessing the enzyme active sites. Having prevented production of pyruvate, α-ketoglutarate, and the oxaloacetate, these two compounds inhibit hepatic gluconeogenesis and consequently, hinder the progression of diabetes.
Significance: This study suggests that HT and HD may be considered as leading compounds for designing safe and effective drugs in management of increased ALT and AST-related disorders specially diabetes.

1. Introduction

Alanine aminotransferase (ALT) (EC 2.6.1.2) and aspartate aminotransferase (AST) (EC 2.6.1.1) are two liver pyridoxal phosphate (PLP) - dependent enzymes involved in gluconeogenesis and amino acid metabolism. They catalyze the intermediary reactions of glucose and protein metabolisms. In these reactions, alanine is converted to pyruvate by ALT and subsequently *via* pyruvate decarboxylase to oxaloacetate or *via* pyruvate dehydrogenase to acetyl coenzyme A. Oxaloacetate and glutamate are the productions of aspartate metabolism accompanied by AST (Scheme 1).

Increased levels of activity of ALT and AST have been observed in a variety of conditions like liver metabolic syndrome, atherogenesis [1, 2] and type I and type II diabetes [3–5]. The use of agents that can inhibit these enzymes may be a new therapeutic approach for the treatment and prevention of the progression of diabetes.

Flavonoids are naturally occurring polyphenols which have been largely studied due to their numerous pharmacological activities such as anticancer, antioxidant and anti-diabetic effects [6–8]. The anti-hyperglycemic and anti-diabetic potential of flavonoids has been largely considered due to their therapeutic potential and low toxicity [9–11].

Hesperetin (HT) and its 7-rutinoside form, hesperidin (HD), are two *citrus* flavanones, a subtype of flavonoids, whose useful biological properties such as anti-inflammation, antioxidant, anticancer, anti-cardiovascular disease [2,12–14] and anti-hyperglycemic effects [15,16] have been demonstrated previously. They also found to be antidiabetic flavonoids through different mechanisms such as lowering serum glucose level, hepatic triglyceride level, and fatty acid oxidation [17]. Constantin et al. have found that these flavonoids exert anti-gluconeogenesis actions through the inhibition of other gluconeogenic enzymes [16]. Since some other flavonoids have been found to inhibit ALT and AST activities [18,19], it can be assumed that HT and HD may be a potent inhibitor of ALT and AST and this may be another mechanism by which these compounds exert their anti-gluconeogenic actions. To examine this

Abbreviations: HT, Hesperetin; HD, Hesperidin; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; NAD^+, Nicotinamide adenine dinucleotide; NADH, Nicotinamide adenine dinucleotide reduced form; PMP, Pyridoxamine 5′-phosphates; PLP, Pyridoxal phosphate; LDH, Lactate dehydrogenase; TCA, Tricarboxylic acid; PDB, Protein Data Bank; ADT, AutoDockTools; IC_{50}, The half maximal inhibitory concentration; SEM, Standard error of the mean.

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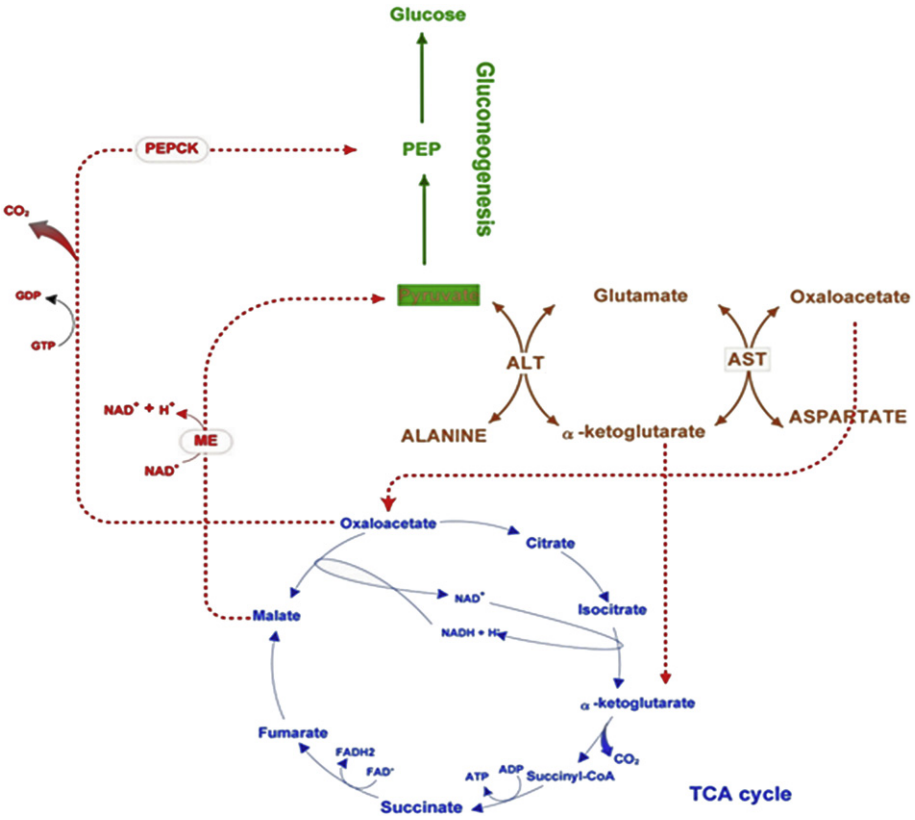

Scheme 1. Schematic representation of gluconeogenesis inhibition by hesperetin and hesperidin. Reactions and pathways are represented in an abbreviated form.

assumption, this study aimed to measure the probable effects of HT and HD on ALT and AST specific activities using a spectrophotometer method and to provide further binding information by molecular docking analysis.

2. Materials and methods

2.1. Chemicals and reagents

The *Citrus* flavonoids hesperetin (HT) and hesperidin (HD) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, Germany. Dulbecco's Modified Eagle Medium (DMEM) was obtained from Gibco laboratories (Paisley, Scotland) to be used as the culture medium.

2.2. Sample preparation

The samples were prepared using five treatments in different concentrations of either HT or HD, each with three replicates. The normal rat liver tissue was purchased from Pasteur institute, Iran. The sample tissue was sliced into 0.5 g pieces, each one minced and added to sterile marked test tubes containing 5 ml of DMEM medium with pH 7.4. HT and HD were dissolved in dimethyl sulfoxide (DMSO) individually to prepare the ligand stock solution. Then sufficient volumes of the each stock were diluted with distilled water to prepare the concentrations of 40, 80, 120, 160 and 200 μM (the final concentration of DMSO was <0.1% in the most diluted solution). Then, 1 ml of either HT or HD was added to each test samples. The control group was provided with no amount of HT or HD. After incubation at 37 °C for 24 h, each mixture was homogenized separately and centrifuged at 4 °C, 3000 RPM and for 5 min to provide liver extract. 0.2 ml of the supernatant was used immediately for ALT and AST activity assay. All of the post-incubation processes were done in 1 day in a controlled temperature room at 25 ± 1 °C.

2.3. Biochemical assay

The ALT and AST activities were determined by monitoring the oxidation of NADH to NAD^+ at 340 nm. The reaction mixtures were prepared in separate tubes using 2.3 ml of L-alanine 61 mM, 0.1 ml of NADH 4.2 mM, 0.1 ml of pyridoxal phosphate 3.4 mM, 0.1 ml of lactate dehydrogenase (LDH) 72,000 U/l and 225 mM of oxoglutarate. Then 0.2 ml of the sample's supernatants obtained from the prepared sample were added to the reaction mixture (the supernatants were stored on ice during the experiment). ALT specific activity was spectrophotometrically analyzed by Desaga, Sarstedt-Gruppe CD60/131700 instrument immediately after 2 mins of incubation at 37 °C. All of the stages were done for AST using the similar mixture, but 2.3 ml of L-aspartate, 0.05 ml of malate dehydrogenase 36,000 U/l were used instead of L-alanine and LDH, respectively. Finally, the results obtained by nonlinear regression were depicted in enzyme activity graphs and used for the half maximal inhibitory concentration (IC_{50}) values calculated using GraphPad software (Prism 6.0 for windows, GraphPad Software, San Diego, California USA, www.graphpad.com).

The total protein concentration in whole liver homogenates was estimated according to the Bradford method [20] with bovine serum albumin as the standard.

2.4. Docking analysis

In order to clarify the probable binding conformations of HT and HD to ALT and AST, molecular docking simulation was performed using AutoDock 4.2 program [21]. Inhibitors' and enzymes' input files were prepared by AutoDockTools (ADT, v. 1.5.6). The structure-data file (.SDF) of HT and HD 3D structures, obtained from the PubChem compounds data bank, were converted to protein data bank (PDB) format by OpenBabel (www.openbabel.org). Polar hydrogens and Gasteiger charges were added and the roots were detected by ADT.