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Inhibitory Effect of Ruta chalepensis Leaf-Derived Component against Alcohol Dehydrogenase

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Abstract Inhibitory activity of active compound isolated from *Ruta chalepensis* leaf was examined against alcohol dehydrogenase and, upon comparison to those of four commercially available compounds (quinoline, quinoline-3-carboxaldehyde, quinoline-3-carboxylic acid, and quinoline-4-carboxylic acid) and 1,10-phenanthroline as alcohol dehydrogenase inhibitor, was characterized as quinoline-4-caboxaldehyde by spectral analyses. Inhibitory effects (IC_{50}) of quinoline-4-caboxaldehyde and quinoline derivatives varied depending on chemicals and concentrations used. The IC_{50} values of quinoline-4-carboxaldehyde, quinoline-3-carboxaldehyde, quinoline, quinoline-3-carboxylic acid, and quinoline-4-carboxylic acid were 0.04, 0.3, 0.8, >1, and >1 mg/mL, respectively. These results suggest inhibitory action of quinoline-4-carboxaldehyde against alcohol dehydrogenase as prospective therapeutics for treatment of alcoholic liver diseases such as alcohol hepatitis and cirrhosis resulting from chronic alcohol abuse.

Keywords: alcohol dehydrogenase, quinoline-4-carboxaldehyde, *Ruta chalepensis*

Introduction

Alcohol dehydrogenase is a key enzyme in ethanol elimination, converting ethanol into acetaldehyde, followed by the concomitant reduction of NAD^+ into NADH. The importance of this enzyme in alcohol metabolism has been shown in many different species. In mammals, alcohol dehydrogenase is the first enzyme in the ethanol-metabolizing pathway and is very active in the liver, playing a significant role in the development of tissue alterations caused by ethanol abuse. Cellular damage occurs not only by the direct effect of ethanol but also through the toxic effect of the first oxidative metabolite, acetaldehyde. In addition, large amount of NADH concomitantly produced changes the redox potential of the cell, thereby disturbing its metabolic features (1-3). Consequently, to elucidate the pathophysiology of alcohol intoxication, the level of alcohol dehydrogenase activity should be determined.

An excess intake of alcohol causes hangover symptoms such as headache and discomfort, which could be due to the effects of ethanol metabolites such as acetaldehyde, acetate, and ketone on the body (4-6). Acetaldehyde, the first metabolite in ethanol metabolism, plays an important role in alcohol toxicity in humans. Many studies have dealt with detoxication effects of herbal medicine, amino acids, and food stuff in treatment of acute alcoholism (7, 8). Plant extracts may be an alternative to currently used alcohol dehydrogenase inhibitors, because they constitute a rich source of bioactive chemicals (9-11). Because many of the plant extracts are largely free from adverse effects and have excellent pharmacological actions, they could lead to the development of possibly safer agents for alcohol dehydrogenase inhibitors. Additionally, some of the plant-derived materials are found to be more effective against alcohol dehydrogenase than current chemotherapeutic agents (9-11). Therefore, much effort has been focused on examining potential use of plants as commercial alcohol dehydrogenase inhibitors or lead compounds. In this study, the active component from *Ruta chalepensis*, a perennial species widely used in folk medicine as an antirheumatic, antispasmodic, and aphrodisiac, as well as to treat snakebites, headaches, and wounds, was isolated by bioassay-guided separation (12). However, relatively little work has been done on the inhibitory activity of *R. chalepensis* against alcohol dehydrogenase despite its excellent pharmacological action. The importance of finding effective inhibitors against alcohol dehydrogenase led us to further investigate these natural compounds. In our study, to develop potentially new safer types of alcohol dehydrogenase inhibitors, the inhibitory effects of leaf-derived materials of *R. chalepensis* against alcohol dehydrogenase were investigated.

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Materials and Methods

Chemicals Alcohol dehydrogenase, bovine serum albumin, β-nicotinamide adenine dinucleotide (NAD), sodium phosphate (Monobasic anhydrous), and Tris-HCl were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Quinoline, quinoline-3-carboxaldehyde, quinoline-3-carboxylic acid, quinoline-4-carboxaldehyde, and quinoline-4-carboxylic acid were provided by Fluka Chemical Corp. (Milwaukee, WI, USA). All other chemicals were of the reagent grade.

Isolation and identification The leaves (3.0 kg) of *R. chalepensis* (family Rutaceae) purchased commercially were dried in an oven at 50°C for 3 days, finely powdered, extracted twice with methanol (15 L) at room temperature,

and filtered (Toyo filter paper No. 2, Toyo Roshi, Japan). The combined filtrate was concentrated *in vacuo* at 45°C to yield crude extract (8.5%, dry weight basis). The extract (20 g) was sequentially partitioned into hexane (2.4 g), chloroform (3.5 g), ethyl acetate (2.7 g), butanol (3.1 g), and water-soluble (8.3 g) fractions for subsequent bioassay. The organic solvent fractions were concentrated to dryness by rotary evaporation at 45°C, and the water fraction was freeze-dried.

The chloroform fraction (40 g) was chromatographed on a silica gel column (Merck 70-230 mesh, 550 g, 70 × 6.0 cm i.d.). Glass column (with PTEE end plate attached) was used selectively according to the quantity of sample. When a sample could not be dissolved by the developing solvent, it was adhered with a small amount of silica gel after dissolving it with methanol to perform chromatography and successively eluted with a gradient of chloroform/methanol (0, 10, 20, 30, 50, and 100%). The active 30% fraction was chromatographed on a silica gel column and eluted with petroleum ether/chloroform (9:1). The active twenty-three fractions were collected and analyzed by TLC (chloroform/methanol, 10:1). Fractions with similar TLC (Thin layer chromatography, SIL G/UV 254, 0.25 mm, Macherey-Nagel, Germany) patterns were combined. The active fraction was chromatographed on a silica gel column and eluted with petroleum ether/chloroform/methanol (20:20:1). For further separation of the biologically active substances, a Waters Delta Prep 4000 HPLC consisting of a Bondapak C_{18} (Waters) column (300 × 39 mm i.d.) was used with a methanol/water gradient (30:1) at a flow rate of 3.5 mL/min and UV detection at 294 nm. Finally, one potent active principle was isolated from the 30% methanol/water fraction. Structural determination of the active isolate was performed through spectroscopic analysis. ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a Bruker AM-500 spectrometer (Rheinspettem, Germany), with chemical shifts given in parts per million. Ultraviolet spectra were obtained on a Waters 490 spectrometer, and mass spectra were obtained on a JEOL JMS-AX 302 WA spectrometer (Tokyo, Japan).

Enzyme and reagent preparation A solid preparation of alcohol dehydrogenase from baker's yeast (EC 1.1.1.1, Sigma, USA) was used. Solutions with enzyme concentration of 5.5 units/mL were prepared by dissolving an enzyme preparation in phosphate buffer (pH 7.6). Solid samples and solutions of the enzyme were stored at -20°C. Solutions of NAD^+ were prepared daily from a solid preparation (Sigma) by dissolving accurately weighed amounts in water. Solid samples and solutions of NAD^+ were stored at 4°C. Ethanol rectificate was used to prepare 1 M solutions by diluting exact volumes of 96% ethanol with water. Inhibition test solutions (1, 0.5, 0.1, 0.05, 0.025, and 0.01 mg/mL) were prepared by dissolving accurately weighted amounts of pure sample in 1-2 drops of very pure concentrated methanol and diluting with water (pH 3-4) to the required volume.

Phosphate buffer solution (1.34 mM, pH 7.6) was prepared by mixing 1.34 mM Na_2HPO_4 and 1.34 mM NaH_2PO_4 solutions and adding 0.1 M NaOH to the required pH value. Tris-HCl buffer solution (50 mM, pH 8.8) was prepared by dissolving accurately weighed amounts of Tris-(hydroxymethyl)-aminomethan (Sigma) in H_2O and adding 0.1 M HCl to the required pH value. All reagents used were of analytical grade. For preparation of all aqueous solutions, doubly distilled water purified by water purification system "JABA UNION-C" (Seoul, Korea) was used.

Enzyme inhibition assay The activity of alcohol dehydrogenase was assayed according to the method described by Shekhovtsova and Zhmaeva (13). Briefly, 50 mM Tris-HCl buffer solution (1.45 mL), NAD^+ solution 1.2 mg/mL (0.01 mL), 100 mM ethanol (0.01 mL), sample solutions (0.01 mL) 100-1,000 or 10-50 mM, or distilled water in the case of a blank experiment, and alcohol dehydrogenase solution 0.011 mg/mL (0.01 mL) were placed in a glass test tube with a ground-glass stopper to the total volume of 1.5 mL. Exactly 90 min after the addition of alcohol dehydrogenase, the absorbance of the solution at 340 nm was measured. The indicator reaction rate was characterized based on the slope of the straight initial part of the kinetic curves with the coordinates, absorbance (A) versus time (S). The calibration graphs were plotted as analyte concentrations. The indicator reaction rate was monitored at 340 nm, corresponding to the maximum absorbance of the reaction product NADH using a spectrophotometer HACH DR/4000 (USA) (=1.0 cm). The pH of buffer solutions was measured by a microprocessor pH-210 (should use journal format throughout text, Korea). For dosage of solutions of the enzymatic process components, alcohol dehydrogenase, NAD^+, ethanol, and sample micropipettes were used. All experiments were performed at room temperature (20-25°C). Although the rate of ethanol oxidation by NAD^+ increases by up to 2% when the temperature increases by 1°C, according to literature data, alcohol dehydrogenase solutions are stable at 45°C.

Results and Discussion

Inhibitory activity of *R. chalepensis* leaf extract materials Fractions obtained from methanol extract of *R. chalepensis* leaf were assessed for inhibitory activity against alcohol dehydrogenase. At 1.0 mg/mL, the chloroform fraction showed 100% inhibition against alcohol dehydrogenase, whereas other fractions exhibited weak or no inhibition (data not shown).

Identification of active constituent The biologically active compound from the fraction was purified by silica gel column chromatography and HPLC, and the isolates were bioassayed. One active isolate showed inhibitory activity. Structural determination of the isolate by spectral techniques characterized the isolate as quinoline-4-carboxaldehyde. The compound was identified based on the following evidence: ($C_{10}H_7NO$, MW, 157.1); EI-MS (70 eV) *m/z* (% relative intensity): M^+ 157 (100), 129 (90), 128 (55), 101 (30), 75 (25), 51 (15); ^{1}H-NMR (CD_3OD, 400 MHz); δ 8.82~8.83 (*d*, 1H, *J*=4 Hz), 8.26~8.28 (*d*, 1H, *J*=8 Hz), 8.02~8.04 (*d*, 1H, *J*=8 Hz), 7.76~7.77 (*d*, 1H, *J*=4 Hz), 7.72~7.75 (*m*, 1H), 7.59~7.63 (*m*, 1H), 6.13 (*s*, 1H); ^{13}C-NMR (CD_3OD, 100 MHz); 151.0, 149.0,

148.1, 130.6, 129.5, 127.9, 127.2, 126.0, 119.1, 95.7.

Compare isolated compound with isoquinoline alkaloids The inhibitory activity of quinoline-4-carboxaldehyde isolated from *R. chalepensis* leaves against alcohol dehydrogenase was examined and compare to that of four commercially available quinoline derivatives (quinoline, quinoline-3-carboxaldehyde, quinoline-3-carboxylic acid, and quinoline-4-carboxylic acid) and 1,10-phenanthroline, which has been demonstrated to be a potent alcohol dehydrogenase inhibitor *in vitro* (Fig. 1). Inhibitory response varied with the chemicals and concentrations used. At 1.0 mg/mL, quinoline-4-carboxaldehyde completely inhibited (100%) alcohol dehydro-genase, whereas the inhibitory activities of quinoline, quinoline-3-carboxaldehyde, quinoline-3-carboxylic acid, and quinoline-4-carboxylic acid were 56, 84, 28, and 29%, respectively. On the basis of their effective inhibitory activities, the inhibitory effect was determined at decreasing concentrations. At the concentration of 0.1 mg/mL, only quinoline-4-carboxaldehyde showed over 90% inhibition, whereas the remaining samples (quinoline, quinoline-3-carboxaldehyde, quinoline-3-carboxylic acid, and quinoline-4-carboxylic acid) exhibited weak inhibition, ranging from 15 to 42% (Fig. 2).

The IC_{50} values of quinoline-3-carboxaldehyde and quinoline-4-carboxaldehyde were 0.3 and 0.04 mg/mL, whereas those of quinoline, quinoline-3-carboxylic acid, and quinoline-4-carboxylic acid were 0.8, >1.0, and >1.0, respectively, with those of quinoline-3-carboxaldehyde and quinoline-4-carboxaldehyde being approximately 2.67 and 20 times lower than that of quinoline. The IC_{50} value of quinoline-4-carboxaldehyde was over 25 times lower than those of quinoline-3-carboxylic acid and quinoline-4-carboxylic acid, whereas 1,10-phenanthroline (IC_{50}, 0.001 mg/mL) was 40 times more potent than quinoline-4-carboxaldehyde (Fig. 3). These results indicate that inhibitory activity against alcohol dehydrogenase was much more pronounced in carboxaldehydated quinolines than in the monoquinoline and carboxyl acids. However, the factor involved in the inhibition of the alcohol dehydrogenase activity by the carboxaldehyde quinoline group is yet unclear.

Plant extracts and phytochemicals are potential alternatives to synthetic inhibitors against alcohol dehydrogenase activity (7-11, 14). Lee and Lee (15) screened natural products for inhibition or activation of alcohol dehydrogenase activity and found methanolic extracts of *Pueraria thunbergiana* (61.2 μg/mL), *Glycyrrhiza uralensis*

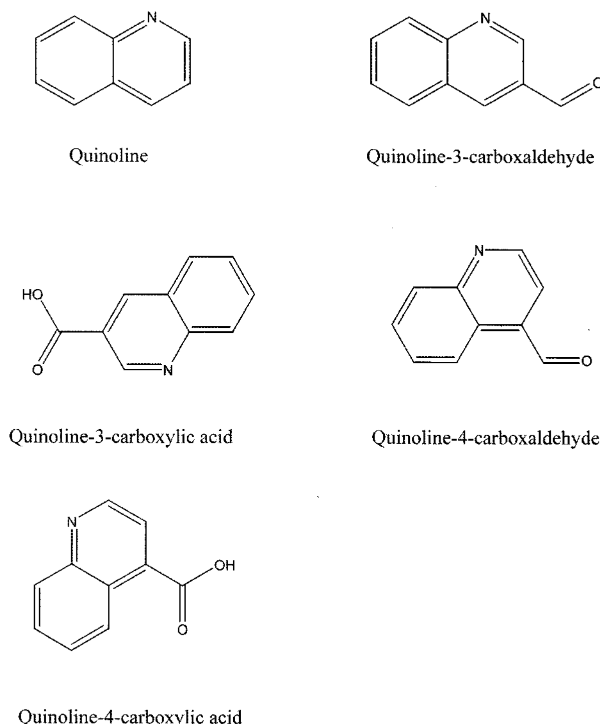

Fig. 1. Structure of quinoline-4-carboxaldehyde isolated from *Ruta chalepensis* leaves and isoquinolines.

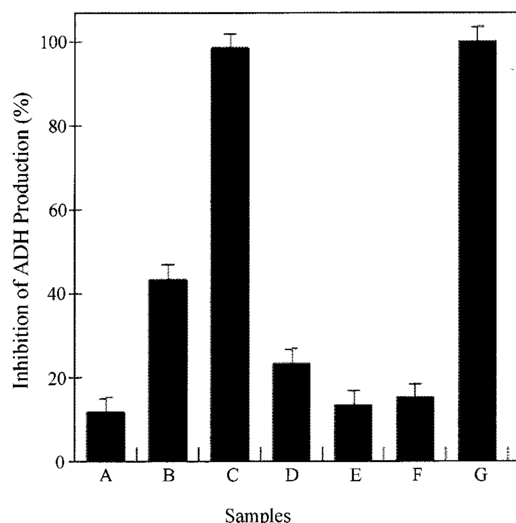

Fig. 2. Alcocol dehydrogenase inhibitory activity of isolated compound and isoquinoline alkaloids at a 0.1 mg/mL. A, control; B, Quinoline-3-carboxaldehyde; C, Quinoline-4-carboxaldehyde; D, Quinoline; E, Quinoline-3-carboxylic acid; F, Quinoline-4-carboxylic acid; G, 1,10-phenanthroline.

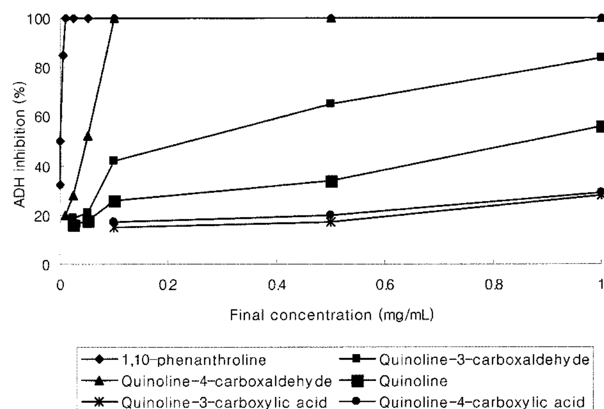

Fig. 3. Inhibitory effect and IC_{50} (mg/mL) of the isolated compound and quinoline derived compounds against alcohol dehydrogenase. 1,10-phenanthroline, 0.001 mg/mL; quinoline-3-carboxaldehyde, 0.3 mg/mL; quinoline-4-carboxaldehyde, 0.04 mg/mL; quinoline-3-carboxylic acid, >1.0; quinoline-4-carboxylic acid, >1.0

(105.0 µg/mL), *Cinnamomum cassia* (7.0 µg/mL), *Rheum undulatum* (36.7 µg/mL), *Morus alba* (106.2 µg/mL), *Chrysanthemum indicum* (112.2 µg/mL), and *Scutellaria baicalensis* (122.5 µg/mL) significantly inhibited alcohol dehydrogenase. In this study, the active component isolated from *R. chalepensis* leaves was identified as quinoline-4-carboxaldehyde. Studies showed *R. chalepensis* leaf-derived materials have antirheumatic, antispasmodic, and aphrodisiac activities (12). It might be expected that the active components isolated from *R. chalepensis* leaves would have some pharmacological actions against hangover.

Alcohol dehydrogenase inhibitors interfere with the metabolism of alcohol by minimizing the generation of its primary metabolite acetaldehyde, thus prolonging the action of alcohol (16, 17). Evidence from both alcohol and acetaldehyde studies demonstrate that each compound is capable of independently producing hazard effects in different organ systems. However, it remains to be determined whether alcohol or acetaldehyde produces specific or differential injurious effects associated with fetal alcohol syndrome. In the presence of alcohol metabolism, the application of alcohol dehydrogenase inhibitor prevents the accumulation of acetaldehyde (18). In this study, the present results are less likely to be attributed to the actions of acetaldehyde, because the majority of the metabolic pathways responsible for the conversion of alcohol into acetaldehyde were blocked by the administration of quinoline-4-carboxaldehyde. These results lend support to the notion that there were only trace amounts of acetaldehyde formed in the presence of the alcohol dehydrogenase inhibitor, and this negligible amount can be metabolized rapidly and effectively into acetate during this neonatal stage.

In conclusion, our results indicate that *R. chalepensis* leaf-derived materials have an inhibitory effect *in vitro* against alcohol dehydrogenase. Results of our limited data and some earlier findings suggest quinoline-4-carboxaldehyde as a useful lead compound for hangover and medicinal foodstuff, although *in vivo* efficacy and clinical utility remain to be evaluated.

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