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# Acetylcholine Esterase Inhibitors in *Rhodiola rosea*

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## Abstract

The alcohol extract or *Rhodiola rosea* has been shown to cause 42 ± 3.2% inhibition of acetylcholine esterase (AChE) when tested at 10 g/L. This AChE inhibition provides a physiological explanation for the reported mental and memory enhancing properties of *Rhodiola rosea* extracts. Active guided fractionation indicated a multitude of components which are responsible for this plants AChE inhibition. Two flavonoid glycosides (gossypetin-7-*O*-L-rhamnopyranoside and rhodioflavonoside) were isolated and shown to cause 58 ± 15% and 38 ± 4% AChE inhibition respectively when tested at 5 g/L. In view of this new enzymatic activity and previous clinical work indicating memory and mental enhancing properties with no indication of toxicity, this plant needs to be researched for its potential at treating memory impairing disorders such as Alzheimer's disease.

**Keywords:** *Rhodiola rosea*, gossypetin-7-*O*-L-rhamnopyranoside, rhodioflavonoside, acetylcholine esterase, AChE.

## Introduction

*Rhodiola rosea* L. (*Crassulaceae*) is a medicinal plant which grows throughout North Asia and the mountains of central Europe. For several centuries the plant has been documented as useful in the treatment of a wide range of illnesses including: stimulating the nervous system, decreasing depression, enhancing work performance, eliminating fatigue, preventing high altitude sickness, and healing burns and contusions (Kelly, 2001). More recently *Rhodiola rosea* has been shown to have anti-hypoxic activity (Kurmukov et al., 1986), antioxidant activity (Furmanowa et al., 1998), antiarrhythmic activity (Malsov et al., 1998), anti-prostate cancer and antibacterial activity (Ming et al., 2003), anti-hepatic cancer activity (Udintsev Schakhov, 1991a,b), and the ability to enhance learning and memory (Petkov et al., 1986). *Rhodiola rosea* extract is a valuable therapeutic as it does not exhibit detectable toxicity throughout a concentration range which exceeds concentrations responsible for its many biological activities (German, Ramazanov, 1999; Yoshikawa et al., 1996; Darbinyan, et al., 2000).

One of *Rhodiola rosea*'s traditional Russian uses is to improve memory and mental function. Typically the dried root is extracted in aqueous alcohol and consumed as an alcoholic liquid extract the evening prior to an exam. Crude extracts of *R. rosea* have been shown to increase short and long term memory and improve learning in rats (Petkov et al., 1986). In a clinical study extracts of *R. rosea* has been shown to cause a 50% improvement in neuro-motoric performance when examined using maze tests which measure hand-control and problem solving abilities (Spasov et al., 2000). In a second clinical study, *Rhodiola rosea* extracts were shown to increased mental function as was indicated by an improvement in the speed of word determination, reverse spelling, and sequential substitution (Darbinyan et al., 2000).

Despite the large amount of research which has investigated the therapeutic properties of *Rhodiola rosea*, there is currently no physiological explanation for the reported memory improving activity of this plant's crude extract. Plants which are known to cause acetylcholine esterase (AChE) inhibition such as *Salvia lavandulaefolia* (Spanish Sage) have been shown to have a similar effect at improving memory (Tildesley et al., 2003). In this study we have investigated *Rhodiola rosea* for possible AChE inhibi-

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tion which may explain the effect this plant has on mental performance.

## Materials and methods

### Plant material

*Rhodiola rosea* root was obtained from Kelly Harvey and TSN labs Inc. 6146 South 350 West Murry, UT 84107.

### General experimental procedures

Melting points were determined with a Gallenkamp melting point apparatus and are uncorrected. IR spectra were obtained using KBr disks on a BOMEM MB-100 spectrophotometer. UV spectra were obtained on a Unicam 8700 series UV/VIS spectrometer. NMR spectra were run on a Bruker Avance-400 MHz spectrometer. EIMS and HREIMS were recorded on a Kratos MS 50 mass spectrometer. Si gel (Merck, 200–400 mesh) was used for column chromatography. Thin-layer chromatography analysis was carried out on silica gel GF254 plates (Merck). Preparative TLC was performed using silica gel 60 GF254 (Merck, 250 µm thickness).

### Extraction

The dried root of *Rhodiola rosea* (300 g) was extracted with hot 95% ethanol (10 L) three times, and the solutions were combined and concentrated *in vacuo* to obtain 50 g of residue. The ethanolic extract was dissolved in water, which was fractionated by liquid-liquid partition with hexanes (three times, each 300 mL), ethyl acetate (six times, each 300 mL), and *n*-butanol (six times, each 300 mL) to yield a hexanes soluble portion (0.2 g), an EtOAc soluble portion (0.6 g), an *n*-butanol soluble portion (15 g), and a remaining water soluble portion (28 g).

### Acetylcholine esterase inhibition

The rate of the acetylcholine esterase-mediated hydrolysis of acetylthiocholine was determined by measuring the rate of production of free sulfur groups produced as acetylthiocholine was hydrolyzed to thiocholine as described earlier (Ellman et al., 1960). A flat-bottom 96-well polystyrene cluster plate (300 µL/well) (Corning, Inc.) was used to contain the enzymatic reaction. Absorbency of the colored ion was detected at 414 nm on a Titertek Multiscan machine. In each well the following solutions were added: 10 µL of 0.01 M 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma Chemical Co.), 250 µL of 6 × 10–7 M acetylthiocholine iodide (Sigma Chemical Co.), 5 µL of test compound dissolved in methanol at a concentration of 630 g/L, 50 µL of 0.5 units/mL of electric eel lyophilized powdered acetylcholinesterase (Sigma Chemical Co.). A 5 µL aliquot of methanol was used for a control. The final concentration of the test compound was 10 g/L. Each reaction was replicated in eight different wells. The rate of the reaction was calculated, and the percent inhibition was determined as the change between the average rate of control and test reactions.

## Activity-guided isolation

The combined butanol extracts (15 g) were chromatographed over Si gel (200–400 mesh) eluted with a chloroform-acetone gradient solvent system. A total of 500 fractions, each 40 mL, were collected. Fractions were pooled according to their similarity in $R_f$ values on thin layer chromatography to give 39 unique fractions, which were tested for the inhibition of acetylcholine esterase. Fractions 5–7 were further purified using Sephadex LH-20 (eluted with methanol) and crystallized at room temperature to yield three compounds (gallic acid, *trans-p*-hydroxycinnamic acid, and *p*-tyrosol) in quantities less then 10 mg each. These compounds were identified and described (Ming et al., 2003), however due to limited material, they could not be tested for acetylcholine esterase inhibition. Fractions 28 was further separated using Sephadex LH-20 (eluted with methanol) and purified by preparative TLC with the solvent system $CH_2Cl_2$/methanol/formic acid (3.5 : 1 : 0.1) to yield gossypetin-7-*O*-L-rhamnopyranoside (15 mg), and rhodioflavonoside (25 mg).

## Results and Discussion

The crude methanol extract of *Rhodiola rosea* root was shown to cause 42 ± 3.2% inhibition of the acetylcholine esterase (AChE) enzyme when tested at a 10 g/L. This level of inhibition is considered mild when compared to isolated AChE inhibitors such as galanthamine which was shown to cause 99.7 ± 0.1% inhibition when tested at the same concentration and under the same conditions; nonetheless this inhibition explains the reported memory enhancing effects of this plant.

Liquid-liquid fractionation was used to separate the crude *Rhodiola* extract into four fractions: a hexane, ethyl acetate, butanol and a water soluble fraction. The yield and inhibitory activity of each fraction is indicated in Figure 1. Each fraction contained AChE inhibitory properties ranging from 30–83% inhibition. The butanol fraction exhibited the highest level of AChE inhibition and thus was selected for further separation. Using silica gel column chromatography the butanol fraction was separated into 39 unique fractions which were identified using TLC analysis. Upon initial examination nearly all of the 39 column fractions exhibited strong acetylcholine esterase inhibition. The extraction and column fractionation were repeated to confirm that this dispersed activity was not due to contamination or improper separation. Improved resolution of the fraction's activity was obtained by retesting the fractions at 2 g/L as is shown in Figure 2. The dispersed activity present throughout the many

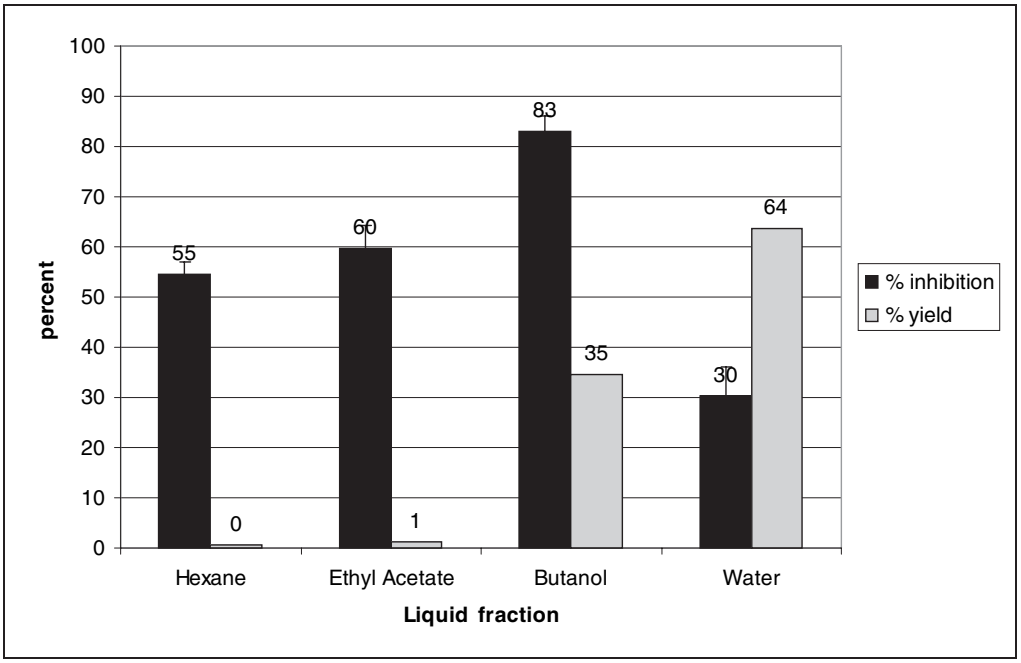


*Figure 1.* Activity of liquid partitions. The acetylcholine esterase inhibition and the corresponding yield of each solvent fraction.

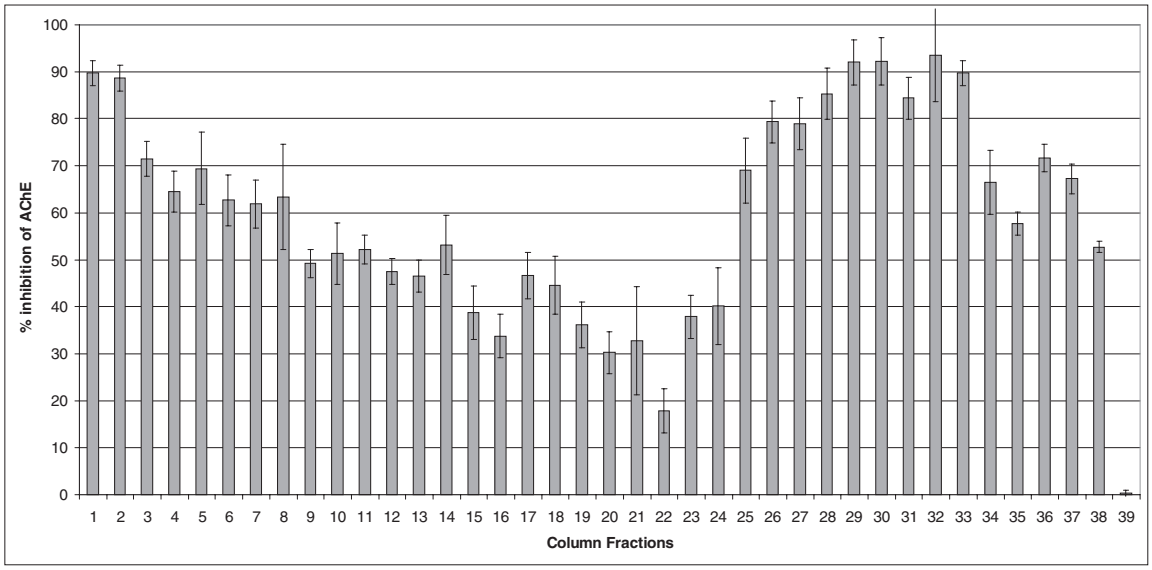


*Figure 2.* Activity of column fractions. The majority of column fractions show high levels of acetylcholine esterase inhibition (tested at 2 g/L).

unique fractions indicates the possibility that this plant's acetylcholine esterase inhibition may be caused by a multitude of chemical constituents.

Fraction 28 was further separated using a Sephadex LH-20. From the subsequent fractions two flavonoid glycosides were purified via preparative TLC. These compounds were identified as gossypetin-7-*O*-L-rhamnopyranoside and rhodioflavonoside by comparing $^{1}H$ and $^{13}C$ NMR, and MS spectra with those reported in the literature (Zepesochnaya et al., 1985; Yoshikawa et al., 1996; Fan et al., 2001; Yu et al., 1993). Both of these isolated compounds exhibited moderate acetylcholine esterase inhibition over various concentrations as is shown in Figure 3.

Due to small fraction sizes further separation of the *Rhodiola* material was not successful. Several compounds were isolated in quantities which were too small for adequate inhibitory testing. Other compounds were isolated and proven to have a similar activity to the identified compounds,

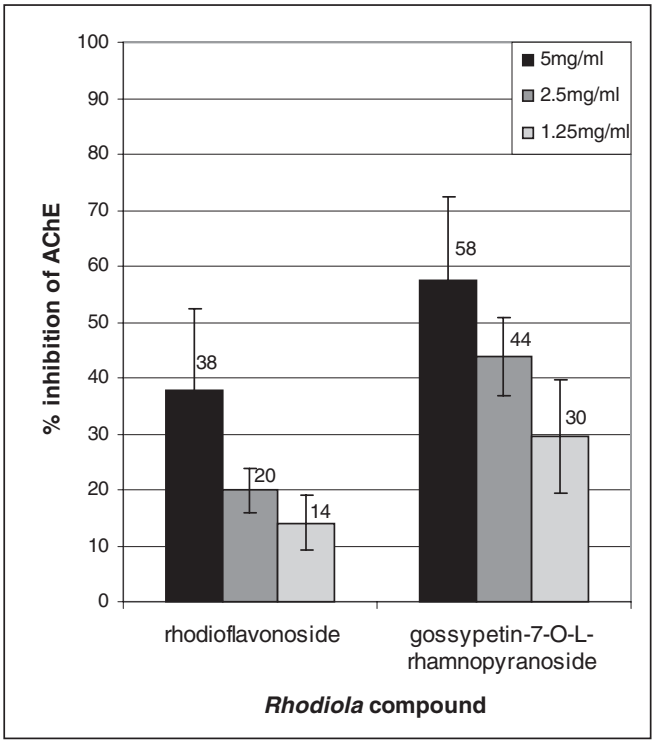


*Figure 3.* Inhibition of AChE caused by *Rhodiola* compounds at various concentrations.

but due to limited material identification of these compounds were not possible.

In conclusion, the alcoholic extract of *Rhodiola rosea* has been shown to cause moderate inhibition of acetylcholine esterase. This plant appears to contain a multitude of different AChE inhibitors including gossypetin-7-*O*-L-rhamnopyranoside and rhodioflavonoside. In view of this plants ability to inhibit AChE and cause memory improvement at levels which do not cause detectable side effects, the extract of *Rhodiola rosea* should be examined for its effectiveness at treating memory impairments such as those caused by Alzheimer's disease.

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