Monoamine Oxidase B Inhibitors from the Fruits of *Opuntia ficus-indica* var. *saboten*

Yong Nam Han, Yeunsu Choo, Young-Chul Lee[1], Young-In Moon[2], Sung-Dae Kim[3], and Jong-Won Choi[4]

Natural Products Research Institute, Seoul National University, Seoul 110-460, [1]Korea Food Research Institute, Seongnam 463-420, [2]Pukcheju Country Rural Community Guidance Center, Pukcheju 695-905, [3]Cactus Village Processing Center, Pukcheju 695-830, and [4]College of Pharmacy, Kyungsung University, Pusan 608-736, Korea

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Three varieties of methyl citrate and 1-methyl malate were isolated from the fruits of *Opuntia ficus-indica* var. *saboten* Makino through *in vitro* bioassay-guided isolation for the inhibition on monoamine oxidase(MAO). The IC_{50} values for MAO-B of 1-monomethyl citrate, 1,3-dimethyl citrate, trimethyl citrate and 1-methyl malate were 0.19, 0.23, 0.61 and 0.25 mM, respectively. However, on MAO-A, their inhibitions showed only marginal activity.

Key words: *Opuntia ficus-indica* var. *saboten*, Monoamine oxidase-B inhibitor, Citric acid methyl ester, 1-Methyl malate

INTRODUCTION

Opuntia ficus-indica var. *saboten* Makino is a tropical or subtropical plant (Cactaceae), which was introduced to seaside area of Cheju Island, Korea. Its fruits and stem have been used on Cheju Island as folk medicines for burns, edema and indigestion. Currently, it is cultivated on Cheju for use in manufacturing health foods such as tea, drinks, and noodles. For utilization as a food material, the color stability of the fruit (Kim *et al.*, 1995; Chung *et al.*, 1996), their composition (Lee *et al.*, 1997), and the quality of wet noodles using its powder (Lee *et al.*, 1999) have been previously investigated.

Several compounds from the cactus have been isolated. Piscidic acid, indicaxanthin in the fruits (Impellizzeri *et al.*, 1972), oligosaccharides in the partially hydrolyzed mucilage (Mcgravie *et al.*, 1981), neobetanin as a minor constituent in the petals (Alard *et al.*, 1985) and two flavonols in the fruits (Jeong *et al.*, 1999) were found in the cactus.

Recently, the ethanol extract of its stem was proven to show anti-inflammatory and analgesic actions in carrageenan-induced paw edema test in rats and acetic acid-induced writhing test in mice, respectively (Park *et al.*, 1998). However, an active principle for the actions was not isolated.

Correspondence to: Yong Nam Han, Natural Products Research Institute, Seoul National University, Seoul 110-460, Korea
E-mail: snake@snu.ac.kr

In order to isolate pharmacologically active constituents from the cactus, we screened several bioassays including antithrombotic, anticoagulant, dopamine β-hydroxylase and monoamine oxidase (MAO) activities. Among these, it was found that the fruits and stem of the cactus inhibit MAO activity. This paper addresses the isolation of well known-but not previously reported in this plant-organic acid methylesters that show inhibitory activity against MAO-B from the methanol extract of fruits.

MATERIALS AND METHODS

General experimental procedures

Melting points were determined with a Mitamura-Riken melting point apparatus and were uncorrected. IR spectra were recorded in KBr using a Jasco FT/IR-5300 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were obtained in CD_3OD or in $CDCl_3$ on a Varian Gemini 2000 spectrometer operating at 300 and 75.5 MHz, respectively. The chemical shifts were reported in parts per million, and the coupling constants (J values) were in Hertz. MS measurement was performed on a Hewlett Packard 5989B Mass spectrometer. TLC analyses were carried out on precoated silica gel F_{254} plates (E. Merck, Darmstadt). The adsorbent used for column chromatography was silica gel 60 (70-230 mesh ASTM, E. Merck, Darmstadt). The solvent system used for TLC was $CHCl_3$-MeOH (5:1 v/v) solution, and the visualization of the TLC plates was performed using a 254 nm UV lamp and I_2 vapor.

Plant material

The fruits and stems, respectively, of *Opuntia ficus-indica* var. *saboten* Makino (Cactaceae) were obtained in lyophylized powdered form in August 1997 from Cactus Village Processing Center (Pukcheju province), Korea.

Monoamine oxidase (MAO) inhibition assay

The MAO-A activity was determined according to the method previously reported by Ryu *et al.* (1988) using serotonin as a substrate. A reaction mixture containing 0.5 ml of enzyme solution in 10 mM phosphate buffered saline (pH 7.0) and 1 ml of test solution was preincubated at 37°C for 15 min., after which 0.5 ml of 10 mM serotonin creatinine sulfate (Sigma Co.) in a buffer was added. Following incubation at 37°C for 90 min, the enzyme reaction was terminated by heating for 3 min in a 95°C water bath. After centrifugation, 1.6 ml of supernatant was loaded to an Amberlite CG50 (H^+ form) column (0.6 cm × 4 cm). The column was washed with over 40 ml of water and the unreacted substrate was eluted with 3 ml of 4 N acetic acid solution and subjected to spectrophotometrical measurement at 277 nm.

Activity was calculated as follows :

Inhibition % =

$$(A_{sample} - A_{compensate} - A_{control})/(A_{blank} - A_{control}) \times 100$$

The MAO-B activity was also determined according to the method previously reported by Han *et al.*, (1987) using benzylamine hydrochloride as a substrate. The reaction mixture containing 0.5 ml of enzyme solution in the buffer and 1 ml of test solution was preincubated at 37°C for 15 min., after which 0.5 ml of 40 mM benzylamine hydrochloride (Tokyo Kasei Co.) was added. Following incubation at 37°C for 90 min, the enzyme reaction was terminated by adding 0.2 ml of 60% perchloric acid. The reaction product, benzaldehyde, was extracted with 4 ml of cyclohexane and subjected to spectrophotometrical measurement at 242 nm. In the control group, water was substituted for the test solution. In the blank group, the substrate was omitted, but was added after the incubation. To compensate the test solution's own absorbance, the substrate was omitted in the compensate group. Activity was calculated as follows :

Inhibition % =

$$(A_{control} - A_{sample} + A_{compensate})/(A_{control} - A_{blank}) \times 100$$

Extraction and isolation

The powdered sample of the fruits (4 Kg) was extracted with MeOH (30 L × 4 times) at room temperature for one month. The extract was filtered and the solvent was removed in vacuo. The residue (1.47 kg) was suspended in water 3 L, and partitioned successively with hexane (3 L × 4 times), EtOAc (3 L × 4 times), and BuOH (3 L × 4 times) leaving the water fraction. Each fraction was evaporated in vaccuo to yield the residues of a hexane fr. (74 g), a EtOAc fr. (147 g), a BuOH fr. (419 g) and a water fr. (813 g), respectively. The EtOAc fr. showed the strongest MAO-A and -B inhibitory activities *in vitro* (Fig. 1).

The EtOAc fr. was chromatographed on a silica gel column using stepwise gradient elution with the eluents, $CHCl_3$, $CHCl_3$/MeOH (10:1, 5:1), $CHCl_3$/MeOH/H_2O (75:25:2.5, 15:10:2.5). The eluates with $CHCl_3$/MeOH showed the strongest inhibitory activity on MAO-B and were titled a MAO fr.

The MAO fr. (66.2 g) was applied to a silica gel column using stepwise gradient elution with the eluents, $CHCl_3$, $CHCl_3$/MeOH (50:1, 30:1, 10:1, 5:1), $CHCl_3$/MeOH/H_2O (75:25:2.5, 15:10:2.5). The subfractions were grouped (fr.1- fr.10) according to their TLC pattern. The subfractions fr.1- fr.6 showed a high MAO-B inhibitory activity on MAO-B (Fig. 3).

Repeated chromatography on silica gel afforded compound I (4.5 g) from fr.1 to fr.6, compound II (0.24 g) and III (0.1 g) from fr.3, and compound (0.05 g) from fr.1.

Compound I (1,3-dimethyl citrate) : colorless powder ; mp. 88-90°C; IR ν_{Max}^{KBr} cm^{-1}: 3490, 3430 (OH), 1742 (ester), 1700 (COOH), 1215, 1127, 984 ; EI-MS *m/z* (%): 189 (M^+-OCH_3, 5), 175 (M^+-COOH, 13), 171 (M^+-OCH_3-H_2O, 25), 143 (171-CO, 100); ^{1}H-NMR (CD_3OD) δ: 3.66 (6H, s, $2CH_3$), 2.94 and 2.81 (each 2H, ABq, J= 15.3 Hz, $2 \times CH_2$); ^{13}C-NMR (CD_3OD) δ: 176.7 (COOH), 172.2 ($COOCH_3$), 74.3 (C), 52.6 (CH_3), 44.2 (CH_2)

Compound II (1-methyl malate) : colorless powder; IR ν_{Max}^{KBr} : 3443 (OH), 3110 (COOH), 1742, 1711, 1269, 1221, 1182, 1121; ^{1}H-NMR (CD_3OD) δ: 4.49 (1H, dd, J=4.7 & 7.2 Hz, CH), 3.73 (3H, s, CH_3), 2.77 (1H, dd, J=4.7 & 16.0 Hz), 2.65 (1H, dd, J=7.2 & 16.0 Hz); ^{13}C-

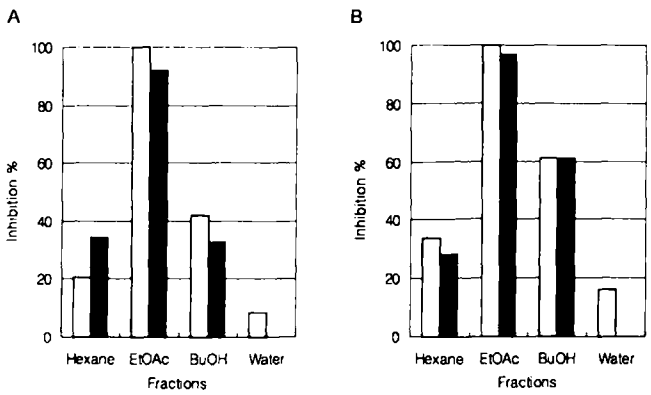

Fig. 1. Monoamine oxidase inhibitory activities of several solvent fractions from the fruits and stems of *Opuntia ficus-indica* var. *saboten*. Each fraction was tested at a concentration of 0.83 mg/ml. A, MAO-A inhibition; B, MAO-B inhibition. □, fruits; ■, stems.

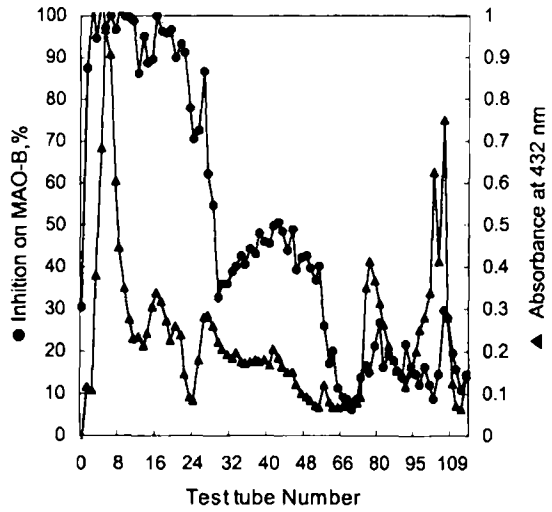

Fig. 2. Comparative monoamine oxidase-B inhibitory profile of eluates from column chromatography on silica gel for the ethylacetate fraction of the fruits of *Opuntia ficus-indica* var. *saboten*. Elution solvents: $CHCl_3$/MeOH (10:1), no 1-16; $CHCl_3$/MeOH(5:1), no 17-45; $CHCl_3$/MeOH/water (3:1:0.1), no 46-69; $CHCl_3$/MeOH/water(15:10:2.5), no 70-120. ●, inhibition % on MAO-B; ▲ absorbance at 432 nm (color of eluate).

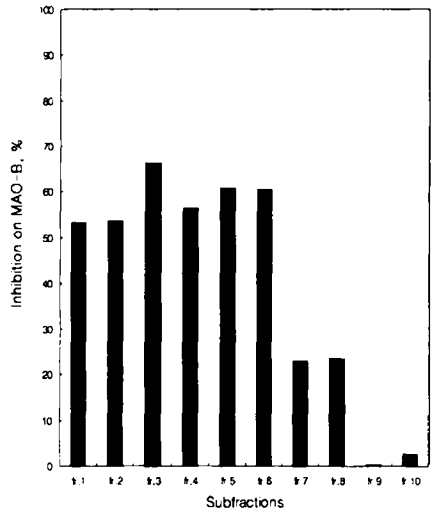

Fig. 3. Monoamine oxidase-B inhibitory activities of the subfractions of MAO fr. Each fraction was tested at a concentration of 0.28 mg/ml.

NMR (CD_3OD) δ: 175.5, 174.1, 68.5 (CH-OH), 52.9 (OCH_3), 39.8 (CH_2)

Compound III (1-monomethyl citrate) : colorless powder; mp. 168°C; IR ν_{Max}^{KBr} cm^{-1}: 3397, 3233, 1745, 1714, 1439, ; ^{1}H-NMR (CD_3OD) δ: 3.75 (3H, s, CH_3), 2.91 and 2.75 (each 2H, ABq, J=15.6 Hz, $2\times CH_2$)

Compound IV (trimethyl citrate) : colorless crystal; IR ν_{Max}^{KBr} cm^{-1} :3484, 1744, 1730, 1209, 1128, 1076, 990 ; ^{1}H -MR ($CDCl_3$) δ: 3.79 (3H, s, OCH_3), 3.65 (6H, s, $2OCH_3$), 2.87 and 2.76 (each 2H, ABq, J=15.5 Hz, $2\times CH_2$)

RESULTS AND DISCUSSION

The EtOAc extract from the cactus fruits showed higher inhibition on both of MAO-A and -B as compared to the other solvent extracts (Fig. 1). When the EtOAc extract was chromatographed over silica gel, the eluates with $CHCl_3$/MeOH (10:1 and 5:1) showed a high inhibition on MAO-B (Fig. 2). Rechromatography of the eluates (test tube numbers 2 to 66) over silica gel yielded 10 subfractions. The higher inhibitory activities on MAO-B were found in the subfractions from fr.1 to fr.6, from which compound I was obtained with a high yield. Repeated chromatography of the maternal solution of each subfraction afforded compound IV from fr.1, compound II and III from fr.3 and additional compound I from fr.4 to fr.6.

Compound I was not positive in Liebermann-Burchard reaction, ninhydrin test or Pauly reaction. Its IR spectrum showed the presence of OH (3490, 3430 cm^{-1}), ester (1742 cm^{-1}) and COOH (1700 cm^{-1}) groups. Two methyl ester groups at δ 3.66 (6H, s) and one pair of AB quartet at δ 2.94 and 2.81 (J=15.3 Hz, $2\times CH_2$) were found in the ^{1}H-NMR spectrum. The ^{13}C-NMR spectrum showed five types of carbon peaks at 176.7 (COOH), 172.2 ($2\times \underline{C}OOCH_3$), 74.3 (C), 52.6 ($COO\underline{C}H_3$) and 44.2 ($CH_2$). Thus, compound I was established as 1,3-dimethyl citrate. Its mass spectrum showed fragment peaks at *m/z* 189 (M^+-OCH_3), 175 (M^+-COOH), 171 (M^+-OCH_3-H_2O) and 143 (171-CO), confirming the above structure to be correct.

The AB quartet system of methylene in the ^{1}H-NMR spectrum was also found in compound III at δ2.91 and 2.75 (each 2H, J=15.5 Hz, $2CH_2$) and compound IV at δ2.87 and 2.76 (each 2H, J=15.5 Hz, $2CH_2$). One methyl ester group at δ3.75 (3H, s, CH_3) was found in the ^{1}H-NMR spectrum of compound III, and the one methyl ester group at δ3.79 (3H, s, CH_3) and two methyl ester groups at δ3.65 (6H, s, CH_3) in the spectrum of compound IV indicated that compound III is 1-monomethyl citrate and compound IV is trimethyl citrate.

Compound II was not positive in Liebermann-Burchard reaction, ninhydrin test or Pauly reaction. Its IR spectrum showed the presence of OH (3443 cm^{-1}), COOH (3110 and 1710 cm^{-1}) and ester (1742 cm^{-1}), which were confirmed in its ^{13}C-NMR spectrum at δ175.5 (COOH), 174.1, 52.9 ($COOCH_3$), and 68.5 (CH-OH). Its ^{1}H-NMR spectrum showed one methyl ester group at δ3.73 (3H, s, 1-$COOCH_3$) and three kinds of couplings between one CH at δ4.49 (1H, dd, J=4.7 & 7.2 Hz), CH_2 at 2.77 (1H, dd, J=4.7 & 16.0 Hz) and one CH_2 at δ2.65 (1H, dd, 7.2 & 16.0 Hz). This data indicated that compound II was 1-methyl malate.

In the stems of this cactus, the spots corresponding to the four compounds were observed on TLC, so no additional investigation was done on the stem extract.

Table I. Monoamine oxidase inhibitory activities of the isolated compounds

Compounds	IC_{50} (mM)*	
	MAO-A	MAO-B
Citric acid dimethylester (I)	> 1.5	0.23
Malic acid monomethylester (II)	> 2.0	0.25
Citric acid monomethylester (III)	> 2.0	0.19
Citric acid trimethylester (IV)	> 1.5	0.61
Sodium Citrate ($2H_2O$ hydrate)	> 330	> 330
Iproniazid**	0.026	-
Norharman**	-	0.01

*Data from 3 experiments of each duplicate.
**Each is a specific known inhibitor for MAO-A or MAO-B.

The *in vitro* MAO inhibitory activities of these four compounds were tested (Table I). While the four compounds showed weak MAO-A inhibition activities, their MAO-B inhibitory activities were stronger; their IC_{50} values were determined 0.25 mM for 1-methyl malate, 0.19 mM for 1-monomethyl citrate, 0.23 mM for 1,3-dimethyl citrate, and 0.61 mM for trimethyl citrate. Regarding citric acid methylesters, the more free carboxylic acid the compound has, the stronger activity it shows. However, citric acid, which has three free carboxylic acids showed an IC_{50} value on MAO-B over 330 mM concentration (Table I). So, the methoxy group in citric acid methylesters seems to be critical for the showing of inhibitory activity on MAO-B.

Citric acid methylesters, particularly dimethylester, are major components in the MAO fraction found in the fruit of *Opuntia ficus-indica* var. *saboten*. Trimethyl citrate has been reported in other plants, but 1,3-dimethyl citrate and 1-monomethyl citrate have not been previously reported. The present study indicates that these molecules including 1-methyl malate have MAO-B inhibitory activities, and that other biological activities or industrial usages of these molecules may be found through further investigation.

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