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Cholinomimetic Properties of a Water-Soluble Fraction from Mulberry Leaves in Rats

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Abstract – The present study examined effects of a water-soluble fraction from mulberry leaves (ML water fraction) on the circulatory and autonomic nervous systems, which were compared with those of acetylcholine (ACh) used as a reference drug in order to elucidate its mechanism of action. Intravenous administration of ACh or a ML water fraction produced temporary depressor and tachycardiac responses in a dose-dependent manner in unrestrained, conscious Sprague-Dawley rats. The systemic hemodynamic effects of ACh and a ML water fraction were almost completely blocked by pretreatment with atropine, a muscarinic antagonist. The depressor responses to ACh and a ML water fraction were slightly enhanced and prolonged by pretreatment with neostigmine, an anticholinesterase, whereas the tachycardiac responses were remarkably blocked by pretreatment with pentolinium, a ganglionic blocking agent. *In vitro* experiments using the ileum isolated from rats showed that ACh and a ML water fraction increased ileal contractility in a dose-dependent manner. The increases in ileal contractility were also completely abolished in the presence of atropine. Finally, the specific binding of [^{3}H]quinuclidinyl benzilate, a muscarinic antagonist, to rat cortical synaptic membranes was inhibited by a ML water fraction in a concentration-dependent manner with an IC_{50} value of 9.5 mg/ml. The results suggest that the effects of a ML water fraction are mediated through direct stimulation of muscarinic cholinergic receptors by unknown cholinomimetic substance(s) contained in that fraction.

Key words □ Mulberrry Leaves, Acetylcholine, Blood pressure, Heart rate, Ileal contractility, Muscarinic receptor

The mulberry tree (*Morus alba* L.) has long been used for the prevention or treatment of various disease conditions including inflammation, diabetes, hypertension, anxiety and insomnia in traditional Oriental herbal medicine (Han, 1989).

Moran A, a glycoprotein, isolated from aqueous methanol extract of the root bark of the mulberry tree was shown to produce hypoglycemic effects in normal and alloxan-induced hyperglycemic mice (Hikino *et al.*, 1985). In addition, water extract (Ko and Shin, 1977), and mulberrofuran F and G (Fukai *et al.*, 1985) isolated from ethyl acetate extract of the root bark of the mulberry tree produced hypotensive effects in rabbits. Phenolic constituents of that root bark were also shown to inhibit cyclic-AMP phosphodiesterase (Nikaido *et al.*, 1984).

Recently, it was shown that hot water extract and its component N-containing sugars from mulberry leaves (ML, Mori folium) produced hypoglycemic effects and potentiated pilocarpine-induced saliva secretion in streptozotocin-induced diabetic mice (Chen *et al.*, 1995). It was in good agreement with the result obtained by our group that a water-soluble fraction from ML (ML water fraction) also produced hypoglycemic effect in alloxan-induced hyperglycemic mice (Lee *et al.*, 1995). Both hypertension and diabetes, especially type II non-insulin dependent diabetes mellitus, have been suggested to be closely associated with insulin resistance (Ferrannini *et al.*, 1987). It is also known that hypertension is one of the most important secondary risk factors in micro-vessel symptoms of diabetes (Choi, 1992). Since various extracts from the mulberry tree were shown to have antidiabetic and hypotensive effects, the present study examined the effects of a ML water fraction on systemic hemodynamics in unrestrained, conscious rats and further characterized its pharmacological action using various *in vitro* techniques in order to elucidate its mechanism of

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action.

MATERIALS AND METHODS

Animals and materials

Male Sprague-Dawley rats weighing 250-300 gm were purchased from Daehan Laboratory Animal Research Center Co., Ltd. (Daejeon, Korea). The rats were housed, before use, in plastic group cages (3-4/cage), under controlled conditions of temperature (22-24°C), humidity (50-60%), and light/dark cycles (12-hour) and maintained *ad libitum* on food and water. The rats were allowed 5-7 days for adaptation before the surgical procedures were performed.

Mulberry leaves were kindly provided by the Korea Agriculture Development Administration. Acetylcholine (ACh) chloride, atropine methyl nitrate, neostigmine bromide, pentolinium ditartrate and heparin were purchased from Sigma Chemical Company (St. Louis, MO). Isoflurane was purchased from Choong Wae Pharmaceutical Company (Seoul, Korea). [^{3}H]Quinuclidinyl benzilate (43.5 Ci/mmol) was purchased from Du Pont Company (Boston, MA).

Preparation of a ML water fraction

A ML water fraction was prepared according to the standard extract preparation method. In brief, powdered ML was decocted in 80% methanol for at least 3 hr for extraction. The extract was dried by evaporation and lyophylization, and then further purified using 50% methylene chloride, 50% ethyl acetate and 50% butanol with each step recovering a water-soluble fraction. The final water-soluble fraction was used as a sample in the experiments.

In vivo experiments on mean arterial pressure (MAP) and heart rate (HR)

For the mean arterial pressure measurement and sample injection, the femoral artery and vein were catheterized under isoflurane anesthesia using the method described by Jin and Rockhold (1991). The animal was allowed to recover from surgery for 2 days and was then used for further experimentation.

On the experiment day, the rats were placed in separate plexiglass experimental cages (10"long × 3.5"wide × 3"deep). The arterial catheter was connected to a 23 gauge connector and the venous catheter was connected to a 30 gauge connector for extension of the tubings. The arterial catheter was then connected to a Cobe pressure transducer (Cobe Laboratory, Inc., Lakewood, CO) and a polygraph (model 7H, Grass Instrument Co., Quincy, MA) that was also connected to a Grass tachograph (model 7P4K) in order to detect heart rate. When the rats were stabilized, test materials were injected intravenously through the venous catheter. The rats were first treated with saline vehicle to obtain control responses, followed by administration of Ach or a ML water fraction. Approximately 10 min later, when the responses to Ach or a ML water fraction returned to pretreatment levels, different blockers were injected. Then, ten minutes later additional ACh or a ML water fraction was injected again. Changes in blood pressure and heart rate produced by the first treatment with ACh or a ML water fraction were compared with changes produced by the second treatment with ACh or a ML water fraction after pretreatments with different blockers.

In vitro experiments with isolated rat ileum

Rats fast overnight were sacrificed. Approximately 1 cm of the ileum was removed. After washing out the contents of the ileum, one side of the isolated ileum was connected to an isometric transducer and the other side to a tissue holder using a 4/0 silk suture. The tissue was placed in an organ bath, and then also connected to the polygraph through the transducer. Twenty ml of Kreb's bicarbonate solution (NaCl 118.4 mM, KCl 4.7 mM, KH_2PO_4 1.2mM, $MgSO_4 \cdot 7H_2O$ 1.2 mM, $CaCl_2 \cdot 2H_2O$ 2.5 mM, $NaHCO_3$ 25 mM, dextrose 10.1 mM, $CaNa_2$·EDTA 0.01 mM, pH 7.4-7.6) was used at 36-37°C and the organ bath was supplied with 95% O_2 + 5% CO_2 (Perry, 1970). Resting tension was at 0.5 gm. Once the movements of the ileum were stabilized, Ach and other reagents were added into the bath. Isometric contractility produced by the reagents was measured by the polygraph.

[^{3}H]Quinuclidinyl benzilate binding assay

Crude synaptic membranes were prepared from the cerebral cortex of male Sprague-Dawley rats according to the method of Zukin *et al.* (1974) with minor modifications. The competitive binding assay was carried out according to the method of Katayama *et al.* (1990) with minor modifications to measure a concentration of the ML water fraction that inhibited the specific binding by 50% (IC_{50} value). In brief, binding of [^{3}H]quinuclidinyl benzilate (QNB, 43.5 Ci/mmol) to synaptic membranes was measured by a filtration assay. The membranes (approximately 0.2 mg of protein) were incubated at 25°C for 1 hr in 50 mM Na^+-K^+ phosphate buffer (pH 7.4) containing 0.1 nM[^{3}H] QNB alone or in the presence of 1 μM atropine methyl nitrate or graded concentrations of the ML water fraction (0.1 - 40 mg/

ml). Total incubation volume was 1 ml. After incubation, the reactions were terminated by rapid filtration through Whatman GF/B glass fiber filters presoaked with 0.05% polyethylenimine using a Brandel M-24R cell harvester. The filters were washed twice with 5 ml of ice-cold buffer, dried and then placed in scintillation vials containing 7 ml of Packard Ultima Gold scintillation cocktail. After shaking and overnight equilibration of the vials, the radioactivity trapped on each filter was measured with a Packard 2000CA liquid scintillation counter. Nonspecific binding was defined as that determined in the presence of 1 mM atropine methyl nitrate. All assays were performed in duplicate. IC_{50} values were determined from the competition binding data using computer-assisted curve fitting with GraphPad Prism 3.0 program.

Statistics

All data were expressed as means ± S.E.M. Statistical significance between the first response to and the second response after pretreatment to Ach or a ML water fraction was analyzed using the paired t-test. Differences were considered to be statistically significant when p value was less than 0.05.

RESULTS

Effects of ACh and a ML water fraction on blood pressure and heart rate

Before treatment with each test material in conscious rats, baseline mean arterial blood pressure (MAP) was 108 ± 6 and 112 ± 11 mm Hg, and baseline heart rate (HR) was 315 ± 14 and 338 ± 18 beats per minute (bpm) in the ACh- and ML water fraction-treated groups, respectively. There was no significant difference in baseline MAP or HR between two groups. Intravenous administration of ACh (0.3-3 μg/kg) and a ML water fraction (5-100 mg/kg) produced instantaneous and transient decreases in MAP but increases in HR in a dose-dependent manner (Fig. 1). When the rats were pretreated with a muscarinic antagonist, atropine methyl nitrate (1 mg/kg, i.v.), depressor and tachycardiac responses produced by ACh (3 μg/kg) and a ML water fraction (50 mg/kg) were almost completely blocked, indicating that the effects were mediated through stimulation of muscarinic cholinergic receptors (Fig. 2). When the rats were pretreated with a peripheral ganglionic nicotinic antagonist, pentolinium ditartrate (5 mg/kg, i.v.), that caused decreases in MAP from 113 ± 7 to 84 ± 8 and from 116 ± 4 to 84 ± 5 mm Hg in the ACh- and ML water fraction-treated groups, respectively, without significantly affecting HR, tachycardiac responses produced by ACh (3 μg/kg) and a ML water fraction (50 mg/kg) were remarkably blocked with slight inhibition of depressor responses (Fig. 3). When the rats were pretreated with an acetylcholinesterase inhibitor, neostigmine bromide (0.1 mg/kg, i.v.), only depressor responses produced by ACh (3 μg/kg) and a ML water fraction (50 mg/kg) were slightly but significantly enhanced (Fig. 4). In addition, recovery time from the depressor responses produced by ACh and a ML water fraction was also delayed from 16.7 ± 3.2 sec to 32.2 ± 3.7 sec (1.9 times) and from 16.3 ± 3.0 to 24.2 ± 3.7 sec (1.5 times), respectively, by the neostigmine pretreatment.

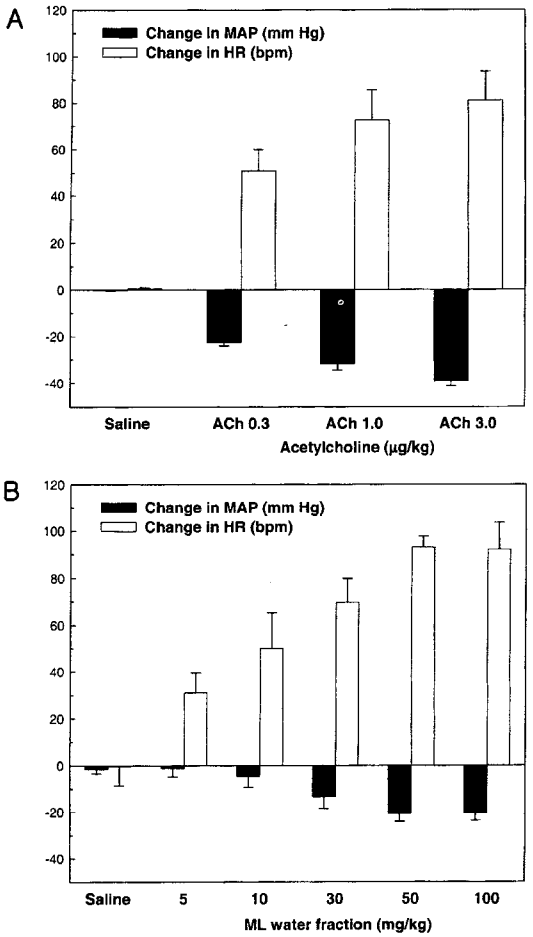

Fig. 1. Dose-related effects of acetylcholine (ACh, top panel A) and a water-soluble fraction from mulberry leaves (MLWF, bottom panel B) administered intravenously on mean arterial pressure (MAP) and heart rate (HR) in conscious Sprague-Dawley rats. Each bar represents a mean ± S.E.M. for four animals.

Effects of ACh and a ML water fraction on contractility of isolated rat ileum

Both ACh (3×10^{-8} -10^{-5} M) and a ML water fraction (0.01-

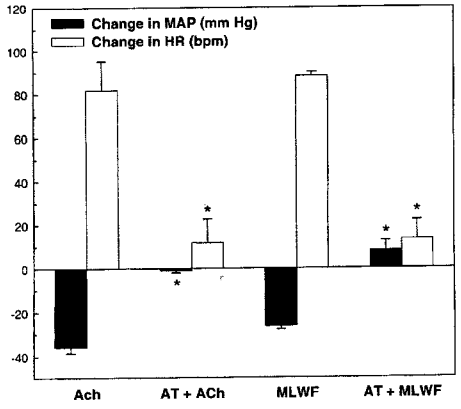

Fig. 2. Effects of pretreatment with atropine methyl nitrate (AT, 1 mg/kg, i.v.) on depressor and tachycardiac responses produced by acetylcholine (ACh, 3 μg/kg, i.v.) and a water-soluble fraction from mulberry leaves (MLWF, 50 mg/kg, i.v.) in conscious Sprague-Dawley rats. Each bar represents a mean ± S.E.M. for four animals. *P<0.05 compared with the control responses to ACh or a ML water fraction.

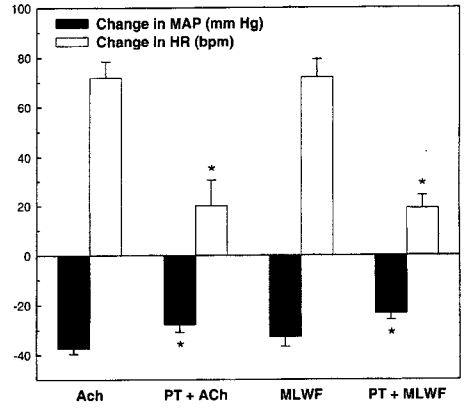

Fig. 3. Effects of pretreatment with pentolinium ditartrate (PT, 5 mg/kg, i.v.) on depressor and tachycardiac responses produced by acetylcholine (ACh, 3 μg/kg, i.v.) and a water-soluble fraction from mulberry leaves (MLWF, 50 mg/kg, i.v.) in conscious Sprague-Dawley rats. Each bar represents a mean ± S.E.M. for four animals. *P<0.05 compared with the control responses to ACh or a ML water fraction.

5 mg/ml) produced increases in ileal contractility in a dose-dependent manner (Fig. 5). Maximum contractility of approximately 2.4 gm was achieved at a concentration of 3×10^{-6} M of ACh. The ML water fraction at a concentration of 5 mg/ml increased ileal contractility by approximately 2.1 gm. However, in the presence of atropine methyl nitrate at a concentration of 5×10^{-5} M, increased ileal contractility produced by ACh (5×10^{-7} M) or a ML water fraction (2 mg/ml) was almost completely blocked, indicating that the effect was mediated through stimulation of muscarinic cholinergic receptors (Fig. 6).

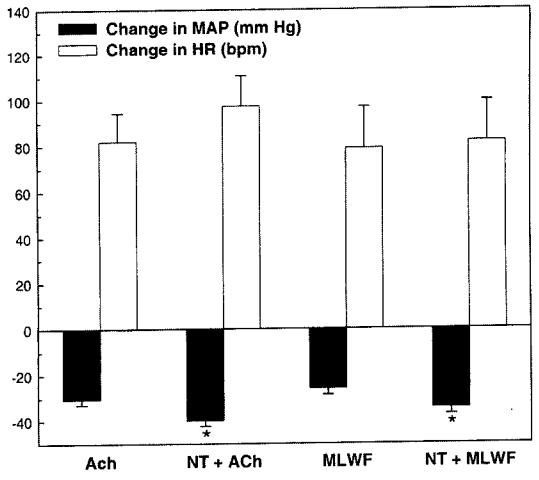

Fig. 4. Effects of pretreatment with neostigmine bromide (NT, 0.1 mg/kg, i.v.) on depressor and tachycardiac responses produced by acetylcholine (ACh, 3 μg/kg, i.v.) and a water-soluble fraction from mulberry leaves (MLWF, 50 mg/kg, i.v.) in conscious Sprague-Dawley rats. Each bar represents a mean ± S.E.M. for seven animals. *P<0.05 compared with the control responses to ACh or a ML water fraction.

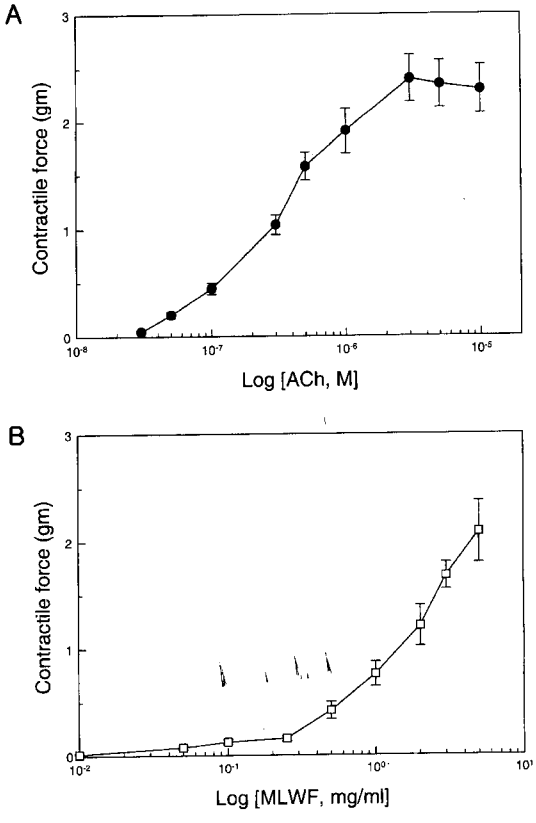

Fig. 5. Concentration-contractile response curves of acetylcholine (ACh, top panel A) and a water-soluble fraction from mulberry leaves (MLWF, bottom panel B) in isolated rat ileum. Each point represents a mean ± S.E.M. for four to five separate experiments.

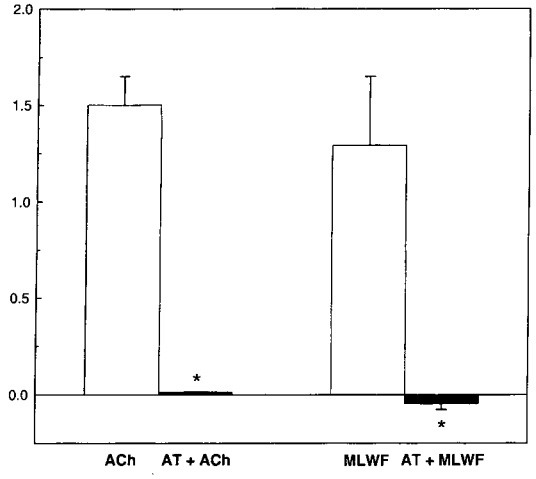

Fig. 6. Inhibition of contractile responses produced by acetylcholine (ACh, 5×10^{-7} M) and a water-soluble fraction from mulberry leaves (MLWF, 2 mg/ml) in the presence of atropine methyl nitrate (AT, 5×10^{-5} M) in isolated rat ileum. Each bar represents a mean ± S.E.M. for five separate experiments. $*P <$ 0.05 compared with the control response to ACh or a ML water fraction.

Effect of a ML water fraction on [^{3}H]QNB binding

The specific [^{3}H]QNB binding to crude synaptic membranes prepared from the rat cerebral cortex was inhibited by a ML water fraction (0.1 - 40 mg/ml) in a concentration-dependent manner (Fig. 7). Approximately 84% of the control specific [^{3}H]QNB binding was inhibited at the maximal tested concentration of 40 mg/ml of the ML water fraction. The measured IC_{50} value was 9.54 ± 0.3 mg/ml.

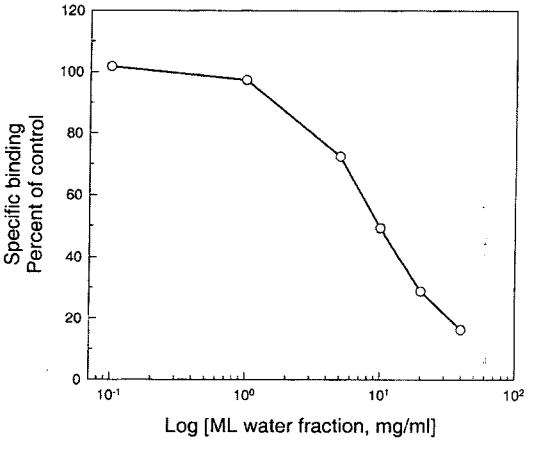

Fig. 7. Effect of a water-soluble fraction from ML (MLWF) on [^{3}H]quinuclidinyl benzilate (QNB) binding to the rat cerebral cortical synaptic membranes. The concentration of [^{3}H]QNB used was 0.1 nM. The results are shown as percent of control (the specific [^{3}H]QNB binding without a ML water fraction). The ML water fraction inhibited muscarinic receptor binding in a concentration-dependent manner. Each point represents a mean ± S.E.M. for five separate experiments.

DISCUSSION

Both intravenously administered Ach and a ML water fraction produced dose-dependent transient hypotension and tachycardia in unrestrained conscious rats. A similar hypotensive response was also observed with a water extract from Mori Radicis Cortex in anesthetized rabbits (Ko and Shin, 1977). Those circulatory effects produced by Ach and a ML water fraction were almost completely blocked by pretreatment with atropine, a muscarinic antagonist, indicating that the effects were mediated through stimulation of muscarinic cholinergic receptors. In addition, since the increases in heart rate were remarkably blocked by pretreatment with pentolinium, a ganglionic blocking agent, at a dose of 5 mg/kg that was shown to completely block the decrease in heart rate induced by norepinephrine, the tachycardiac effects were likely to be mediated through central reflex induced by hypotension in conscious rats (Brown and Taylor, 2001). Pretreatment with neostigmine, an acetylcholinesterase inhibitor, slightly enhanced hypotensive responses and prolonged duration of hypotension produced by ACh and a ML water fraction. Thus, the ML water fraction produced very similar effects to ACh on the systemic hemodynamics.

To further demonstrate the cholinomimetic property of the ML water fraction, effects of Ach and a ML water fraction were examined and compared using isolated rat ileum. Both Ach and a ML water fraction increased ileal contractility in a dose-dependent manner. Effect of a ML water fraction was tested up to the maximal final concentration of 5 mg/ml. Due to the solubility limitation of stock solutions, final concentrations higher than 5 mg/ml could not be tested. As the case in the above circulatory experiment, increased ileal contractility produced by the ML water fraction was completely blocked in the presence of atropine, further confirming that the effect was also mediated through stimulation of muscarinic cholinergic receptors. Thus, the ML water fraction also produced very similar effects to ACh on the ileal contractility, suggesting that the ML water fraction might contain cholinomimetic substance(s).

Finally, to examine whether the ML water fraction contained cholinomimetic substance(s) capable of binding to the muscarinic cholinergic receptors, competitive binding assay was performed using [^{3}H]QNB, a muscarinic cholinergic receptor ligand, and rat cortical synaptic membranes. The ML water

fraction inhibited the specific [^{3}H]QNB binding to crude synaptic membranes in a concentration-dependent manner with an IC_{50} value of 9.54 mg/ml, indicating that the fraction contained cholinomimetic substance(s) directly interacting with the muscarinic cholinergic receptors. The chemical nature of the cholinomimetic sustance(s) remains to be elucidated.

The present study demonstrated that a ML water fraction produced very similar effects to ACh on the systemic hemodynamics and ileal contractility, and contained cholinomimetic substance(s) directly stimulating muscarinic cholinergic receptors. Since vagal nerve stimulation was known to enhance release of insulin from pancreas (Davis and Granner, 2001), it was also possible that the cholinomimetic substance(s) might contribute to the previously reported hypoglycemic effect of a ML water fraction. Future study will be focused on identification of the cholinomimetic substance(s) in the ML water fraction.

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