Synergistic Anti-diabetic Effect of *Cirsium setidens* Combined with Other Plants *in vitro* and *in vivo*

Guo Huifang†, Yunyao Jiang† and Myeong-Hyeon Wang*

Department of Medical Biotechnology, Kangwon National University, Chuncheon 24341, Korea

**Abstract** - The anti-diabetic effect of *Cirsium setidens* water extract and the combinations with *Bletilla striata*, *Cymbidium kanran*, and *Sparganium stoloniferum* Buch.-Ham. ethanolic extracts had been studied. The combination of four extracts (3:1:1:1) showed larger anti-diabetic activity *in vitro* and *in vivo*. It is notable that the single water extract from *C. setidens* exhibited more effective anti-diabetic effect than most of the combinations. We also investigated whether fermentation process was promoted the anti-diabetic activity. The data suggested the fermentation product of combination of four extracts (3:1:1:1) exhibited the strongest activity both *in vitro* and *in vivo*, which was higher than the non-fermented group. The result indicated the fermentation and the appropriate combination of extracts enhanced the anti-diabetes activity.

**Key words** - Anti-diabetes, *Cirsium setidens*, Hypoglycemic, Synergistic

**Introduction**

According to the statistical of World Health Organization (WHO), 347 million people worldwide have diabetes. In 2014, 9% of adults and older had diabetes, and it was the direct cause of death of 1.5 million people in 2012. It is growing into the leading threat of death all over the world. Diabetes mellitus is a chronic disease marked by high levels of glucose in the blood, either because insulin production is inadequate (Type1), or because the cells do to respond properly to the insulin (Type 2), or both. Type 1 diabetes is insulin dependent and the patient cannot live without injecting insulin, and almost 90% is Type 2 diabetes which can be managed by inhibiting the carbohydrate-hydrolytic enzymes like α-amylase and α-glucosidase to reduce the digestion of carbohydrates in order to decrease the postprandial hyperglycemia (Kim et al., 2013; Mitra, 2008; Tiwari and Madhusudanarao, 2002).

Plants are the richest resources of medicine due to its secondary metabolites contain a variety of bioactive compound which have been used in treating long-term diseases like diabetes especially in developing countries for many years, 400 or more plants were demonstrated to have glucose-lowering activity (Choi et al., 2007; Ernst, 1997). *Bletilla striata*, *Cymbidium kanran*, and *Sparganium stoloniferum* Buch.-Ham. had been reported capable of inhibit the α-glucosidase (Xu et al., 2009; Yu et al., 2011). *Cirsium* genus commonly known as thistles, is a perennial and biennial flowering plant used in folk medicine treatment for anti-phlogistic, hemostasis, hypertension, and anti-cancer. *Cirsium setidens*, natively growing in Kangwon do, Korea, has been used in treating hypertension and hepatitis in local residents. However, little research focused on the hypoglycemic effect of *C. setidens*, Kim et al. (2011) reported the plant water extract had no activity on α-amylase and α-glucosidase inhibition activity and an extremely low inhibition effect of ethanolic extract on inhibiting α-glucosidase and α-amylase of 3.0 ± 6.6% and 0.9 ± 5.1%, respectively.

The improved notion of extracting and using the natural product has been reported. in these days Natural products extracted after fermentation attracts researcher’s attention, since the fermentation with some special bacteria provided good quality and activity products while excluding the toxicity from organic solvents, The secondary metabolites produced by microorganisms was obtained as bioactive products (nee’Nigam, 2009). Đorđević et al. (2010) demonstrated that the fermentation increase the antioxidant activity of cereals. Furthermore, Atangwho...
et al. (2012) use the combination of Vernonia amygdalina Dell and Azadirachta indica A. Juss extract to treat the streptozocin-induced diabetic rat and found the combined extract decreased the blood glucose and kept it steady for a long time better than the single extract. Thus, our research focused on the anti-diabetic effect of the combination of C. setidens and other three plant extracts and also investigated if the fermentation increased this effect.

**Materials and Methods**

**Chemicals and reagents**

- p-Nitrophenyl α-D-glucopyranoside (pNPG), dinitrosalicylic acid (DNS), streptozotocin (STZ), starch, α-glucosidase from Saccharomyces cerevisiae, α-Amylase from porcine pancreas, and starch were products of Sigma Aldrich Co., St Louis, USA. Other chemicals and reagents used were of analytical grade and water was distilled.

**Extraction procedure**

The leaves of C. setidens used in this research were harvested in Gangwon do, Korea, Bletilla striata, Cymbidium kanran, and Sparganium stoloniferum Buch.-Ham. were obtained in Korea, and all plant material were dried in shade then milled. The powder was soaked in water or ethanol for 15 h (weight was shown in Table 1.) and the mixture was filtered through filter paper (110 mm; HYUNDAI, Seoul, KOR) to collect the supernatant (C. setidens was extracted by water and other plant were extracted by ethanol). Repeat this step and mix the supernatant as shown in Table 1 then roast the supernatant by vacuum rotary evaporator (CCA-1110; Eyela, Tokyo, Japan) except Fermented Group 8 (FG8). Fermented Group 8 was inoculated Lactobacillus casei and Bifidobacterium bifidum. (1:1) with the density of 10^6 cfu/㎖ after mix the supernatant and fermented for 120 days under 23°C, then evaporated to get the extract.

**α-Glucosidase inhibition assay**

Alpha-glucosidase activity was taken out by the method of by Kim et al. (2005), pre-incubating the mixture of 10 µl extract solution and 40 µl α-glucosidase (0.75 unit/㎖) at 37°C for 10 min, and add 950 µl substrate solution of 4 mM pNPG at 37°C for 20 min incubation (pNPG was dissolved in phosphate buffer of pH 6.9). The reaction was stopped by adding 2 ㎖ of 0.1 M Na₂CO₃. The inhibition activity was determined by measuring absorption at 400 nm and expressed as percentage of blank control. Two groups were used for positive control, Control 1 (C1) was the combination extract of Tilia taquetii C.K.Schneid., Cinnamomum cassia Blume, and grape seed (Patent number: 10-0869443, Korea). Acarbose was used as Control 2 (C2).

**α-Amylase inhibition assay**

This assay was evaluated by a modified method of McCue and Shetty (2004), 50 µl extract solution was mixed with α-amylase (0.3 unit/㎖) of 500 µl (pH6.8) and preincubated at 37°C for 10 min, then 250 µl of 2% starch was added and incubated at 37°C for 5 min. The reaction was stopped by heating at 100°C for 15 min after added 500 ㎕ of DNS (pH 6.8). The mixture solution was cooled down to the room temperature to measuring the absorption at 540 nm.

**Induction of diabetes of rats**

12 adult male (7-9 week, 250-300 g) Sprague-Dawley (SD) rats (Hanlim Animal Lab., Seoul, Korea) was injected 75 mg/kg streptozotocin (dissolved in citrate buffer) into the thigh muscle to induce Type 2 diabetes (Sharma et al., 2014). After one week, blood was collected from tail and measuring the fasting plasma glucose level of rats by Glucotrend monitor (Roche

---

<table>
<thead>
<tr>
<th>Table 1. The combination of C. setidens and other plant</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>G5</th>
<th>G6</th>
<th>G7</th>
<th>G8</th>
<th>FG8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cirsium. setidens</strong></td>
<td>30</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td><strong>Bletilla striata</strong></td>
<td>-</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>7.5</td>
<td>7.5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><strong>Cymbidium kanran</strong></td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>-</td>
<td>7.5</td>
<td>-</td>
<td>7.5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><strong>Sparganium stoloniferum Buch.-Ham.</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>7.5</td>
<td>7.5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

*G1 indicate Group 1, the same as G2, G3, G4, G5, G6, G7, G8, and FG8 indicated the fermented Group 8.*
Hypoglycemic effect on diabetic rats

The diabetic rats were fasted for 12 h and were intraperitoneally injected with 7.0 ml/kg diet food as shown in Table 2. Shortly afterwards, 12 diabetic rats was divided into 4 groups randomly and fed with the mixture of starch of 1 g/kg and extract of 0.5 g/kg, the control group only fed with starch. The blood were collected from each group before feeding (0 min) and at 30, 60, 90, 120, 150, 180, 210 and 240 min after feeding.

Hypolycemic effect on normal rats

The same procedure as measuring the hypoglycemic effect on diabetic rats was carried out on normal male Sprague-Dawley rats. And the test samples were divided into 4 groups with the total amount of 16.

Table 2. The diet for diabetic or normal rats

<table>
<thead>
<tr>
<th></th>
<th>Starch (g)</th>
<th>Glucose (g)</th>
<th>Tween 80 (㎖)</th>
<th>Physiological saline (㎖)</th>
<th>Extracts (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.0</td>
<td>0.29*</td>
<td>1.0</td>
<td>13.0</td>
<td>-</td>
</tr>
<tr>
<td>G1</td>
<td>2.0</td>
<td>-</td>
<td>1.0</td>
<td>13.0</td>
<td>1.0</td>
</tr>
<tr>
<td>G8</td>
<td>2.0</td>
<td>-</td>
<td>1.0</td>
<td>13.0</td>
<td>1.0</td>
</tr>
<tr>
<td>FG8</td>
<td>2.0</td>
<td>-</td>
<td>1.0</td>
<td>13.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*The extract contains glucose of about 29%.

Fig. 1. α-Glucosidase inhibition activity of combination extract and single extract of *C. setidens*.

**Statistical analysis**

All tests were taken out in triplicate (n = 3) and results are expressed in terms of mean ± standard deviation. Statistical analysis was calculated by Excel 2013 (Microsoft, USA).

**Results**

**α-Glucosidase inhibitory activity**

As shown in Fig. 1, a large inhibition activity of all the groups at low concentration was observed in α-glucosidase activity. *C. setidens* water extract (G1) exhibited a rather high level of inhibitory effect, which was higher than the combination with single plant of *Bletilla striata* (G2), *Cymbidium kanran* (G3), *Sparganium stoloniferum Buch.-Ham.* (G4). Moreover, its inhibition activity was also higher than the Control 1 group (C1) of the combination extract of *Tilia taquetii C.K.Schneid.*
Synergistic Anti-diabetic Effect of *Cirsium setidens* Combined with Other Plants *in vitro* and *in vivo*

**Fig. 2.** α-Amylase inhibition activity of combination extract and single extract of *C. setidens*.

*Cinnamomum cassia* Blume, and grape seed and similar with inhibition effect with the Acarbose (C2). However, the G8 and FG8 group had the better inhibition activity than G1, and showed a concentration dependent manner with the highest inhibition activity of α-glucosidase of 1.0 mg/ml.

**α-Amylase inhibitory activity**

Alpha-amylase inhibitory activity was measured by the percentage of different concentration and combination of extracts inhibiting the enzyme catalyze digestion of starch compare to the non-extract group. Thus, from Fig. 2, it is obvious that the *C. setidens* water extract (G1) exhibited an extraordinary inhibition activity than the combination of the single plant extract (G2, G3, G4), but still lower than the Acarbose (C2), the Control 1 group of the mixture extract of *Tilia taquetii* C.K.Schneid., *Cinnamomum cassia* Blume, and grape seed had the lowest inhibition activity, whereas mixture extract of *C. setidens*, *Bletilla striata*, *Cymbidium kanran*, and *Sparganium stoloniferum* Buch.-Ham. ethanolic extract (G8) showed the higher activity than C2, but the fermentation of this mixture (FG8) exhibited even stronger inhibition activity with 80% inhibition activity when the concentration was only 0.01 mg/ml.

**Hypoglycemic on streptozotocin induced Type 2 diabetic rats**

In order to prove the anti-diabetic effect of the extracts, we further investigated the hypoglycemic effect on 12 streptozotocin induced Type 2 diabetic rats. The postprandial level of blood glucose was 290-295 mg/dl, the blood glucose raised to the highest point of all the groups, after fed for 30min and then decreased along with time (Fig. 3). Moreover, the Control group which only fed with the starch increased 120 mg/dl at 60 min, in contrary to the extract groups only increased 85 mg/dl, 78 mg/dl, and 70 mg/dl of G1, G8 and FG8, respectively. In addition, the FG8 group inhibit the blood glucose increase most effectively at the each time point, follow by the G8 and G1.

**Hypoglycemic on normal rats**

To demonstrate the FG8, G8 and G1 were effective hypoglycemic agent (Fig. 4), the same test was carried out on normal rats as the diabetic rats. The postprandial level of blood
glucose was 80-85 mg/dl of normal rats, and this data increasing and decreasing sharply at first 60 min after fed. The Control group increased about 65 mg/dl at 30 min, meanwhile the FG8 group only raised of 30 mg/dl. G1 and G8 raised of 45 mg/dl and 38 mg/dl, respectively. Which indicated both of the three groups inhibit the blood glucose after fed effectively, and the FG8 group was the most effective group.

Discussion

The prevalence of diabetes is increasing worldwide and WHO predict that the diabetes will be the seventh leading cause of death by 2030 (Shaw et al., 2010). Type 1 diabetes is insulin dependent because of the destruction of the insulin-producing beta cells in the pancreas. Type 2 diabetes is noninsulin dependent and occurs because of the genetic or environment factors (Cnop et al., 2005). It manifested as the insufficient of insulin production of pancreatic β cells (Park et al., 2010). One of the strategies of Type 2 diabetes treatment is the inhibition of carbohydrate digesting enzymes like α-amylase and α-glucosidase in order to decrease the absorption gastrointestinal of glucose and eventually lowering postprandial blood glucose level (McCue et al., 2005). α-amylase is capable in catalyzing the hydrolysis of α-1,4-glucosidic bond of starch, glycogen and various oligosaccharides and α-glucosidase further breaks down the disaccharides into monosaccharide, which is directly available for the absorption (Bhandari et al., 2008). The inhibitors of α-glucosidase are employed to achieve better glycaemia control, especially the postprandial hyperglycemia (Kim et al., 2005), since it is involved in the enzymatic action in the surface of brush border membrane of intestine, which ultimately delay the digestion of carbohydrate and diminish the glucose level after meal (Sunil et al., 2009).

In this study, the fermentation product of the mixture of the C. setidens water extract, Bletilla striata, Cymbidium kanran, and Sparganium stoloniferum Buch.-Ham. ethanolic extract (3:1:1:1) was the most effective inhibitor (FG8), followed the non-fermentation of this mixture (G8) and the C. setidens water extract (G1). However, the non-fermentation group (G8) showed a relative higher inhibition effect than neither extract groups (G1, G2, G3, G4, G5, G6, and G7) nor Acarbose (C2) which is well known as an effective inhibitor of the both two enzymes (Fig. 1 and Fig. 2). This suggested the combination usage of the extract improved the activity somehow. Rasoanaivo et al. (2011) reported that the different plant extract working together had pharmacodynamic synergy effect, some component from the other plant may enhanced the activity of the responsible component, or it may protected the responsible component from the degradation of enzymes (Gilbert and Alves, 2003). The administration extracts may causing different result of its bioactivity, some of which enhanced the bioactivity (G8) and some of which may had toxic effects (G2, G3, and G4) (Ezuruike and Prieto, 2014).

FG8 group exhibited the strongest activity not only in vitro enzyme inhibition but also in vivo hypoglycemic effect. The fermentation process could improve the bioactivity had been reported by Đorđević et al. (2010), which may be because of the increasing content of phenolics, folates, lignans and alkyresorcinols after fermentation (Martins et al., 2011; Park et al., 2010). Moreover, the inhibition activity increased compare with G8 due to the other following reasons. Reddy and Pierson (1994) had reported that the fermentation declined the toxic compounds content and nutritional loss and produced a serials product such as α-amylase. The other research demonstrated that the fermentation product increased the sensitivity of insulin in rats (Balan et al., 2002).

The anti-diabetic effect of C. setidens leave extract singly or combination with other plants has been studied. The results suggested the fermentation of the combination of C. setidens water extract, Bletilla striata, Cymbidium kanran, and Sparganium stoloniferum Buch.-Ham. ethanolic extract (3:1:1:1) exhibited the extremely stronger activity both in vitro and in vivo. The
non-fermentation group showed a litter lower activity followed by the single water extract of *C. setidens*, the combination of *C. setidens* and single or double plants. This indicated that, the *C. setidens* water extract is an effective enzyme inhibitor involved in the glucose formation process, but the combination usage can improve or reduce this activity. The more attempts of the interaction worth to be researched and the best proportioning might not 3:1:1:1. Additionally, the fermentation of combination may enhance the effect. We wish that in this manner, the potential therapeutic plants achieve the greatest value in anti-diabetes.

**References**


Sharma, S., M. Choudhary, S. Bhardwaj, N. Choudhary and


(Received 17 November 2015 ; Revised 1 December 2015 ; Accepted 7 December 2015)