IN VIVO STUDY ON ETHANOLIC EXTRACT OF ALLIUM CEPA ON MICE TO EVALUATE THE ANTI-HYPERGLYCEMIC ACTIVITY

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ABSTRACT

As diabetes and blood glucose concerns have been raising, other feasible treatments are now being conducted beside the conventional ones. There has been a shift to the consumption of naturally-derived medicines instead of chemically-derived ones due to novel tendency in medical treatment application. Allium cepa (A. Cepa), a familiar herb used in daily life as a spicy in cooking, was found to have anti-hyperglycemic activity. Extraction using various concentration of ethanol was done to assess the efficiency in total extraction. The 0° ethanol solvent (distilled water) was observed to give the highest efficiency in crude extraction. Total flavonoid and total phenolic content was measured. Although the efficiency in crude extraction of 96° ethanol is lower than those of other concentrations, it flavonoids-releasing and phenolics-releasing capacities are the greatest ones, which are 0.404 % and 11.129 % respectively. The IC₅₀ values of 70° 80°, and 90° ethanolic extract are far higher than that of the control. Then, normoglycemic test and glucose tolerance test were conducted to evaluate the anti-hyperglycemic effect of the A. cepa ’s extract. While the normoglycemic test came up with no significant changes on blood glucose levels of the normoglycemic mice, the glucose tolerance test pointed out the extract’s dosage of 4 mg/kg b.w could considerably reduce blood glucose level of oral-glucose-loading mice compared to negative control administering saline water and positive control administering Metformin and Amaryl. Eventually, since the study partially confirmed the anti-hyperglycemic activity of A. cepa ’s extract, further investigations are recommended.

Keywords: Allium cepa, blood glucose level, anti-diabetes, normoglycemic.

1. INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is an endocrine disorder closely associating with insulin resistance, insufficient insulin secretion, and excessive glucagon secretion with common symptoms such as polyuria, polydipsia, polyphagia, weight loss and blurred vision. According to the latest updated information provided by Diabetes Québec, a non-profit organization assisting people and raise awareness of diabetes for over six decades, current commercial drugs are
divided into 8 classes comprising of depending on their specific targets [1]. In fact, anti-diabetic drugs are unable to completely cure diabetes, but maintaining and stabilizing blood glucose level to lower the complications. For chemically synthetic drug, sides effects, such as skin rash, itching, and weight gain are unavoidable besides their therapeutic ones [2]. In recent years, replacing chemically synthetic drug by herbal treatments as a novel trend has been strongly encouraged due to its high safety, lower cost, and less side effects. Many medicinal herbs which have been traditionally used to treat diabetes are now investigated and evaluated their anti-hyperglycemic activity to provide more scientific evidences [3–5].

*Allium cepa* (A. cepa) or red onion belongs to Alliaceae family. They widely distribute all over the world. For a very long time, they have been prevalently used as foodstuff, condiment, flavoring and folk medicine [6]. *A. cepa* has many therapeutic effects, especially anti-hyperglycemia [7–12]. According to Handbook of Phytochemical Constituent Grass, Herbs and other Economic Plants, there are at least 268 compounds in *A. cepa* [13]. Among those, anthocyanin, a derived subclass of flavonoid, has been proved to reduce risk of T2DM [14]. Besides, high content of flavonoid may serve as the potential sources of antioxidant which captures free radicals and inhibits lipid peroxidation. Since the *A. cepa* possess potential compounds targeting on T2DM-related protein, we conducted experiment to evaluate the anti-hyperglycemic effect of *A. cepa*.

### 2. MATERIALS AND METHODS

#### 2.1. Sample collection

*A. cepa* was collected in Vinh Long (Viet Nam) and authenticated by Agronomy Department - University of Agriculture and Forestry – HCMC (Viet Nam). The healthy albino mice were purchased from The Pasteur Institute – HCMC.

#### 2.2. Sample preliminary preparation

Onion buds were carefully washed with distilled water to remove any type of contamination before being soaked in 70° ethanol to exterminate any enzymes available in the sample. Cleaned samples were left for air-drying. Then, dried samples were thinly sliced and dried in oven at 40 °C for about 94-96 hours until their moisture content reached 8 %. Finally, dried samples were powdered and preserved in desicicator for further use.

#### 2.3. Extraction and concentration

Extraction was processed following the guideline of Adom and Liu [15]. 1 gram powdered onion was extracted 3 times with 10 mL ethanol in various concentrations (96°, 90°, 80°, 70°, 50°, 25°, and 0°) with continuous shaking within 30 minutes for each time. Total extract was centrifuged at 3200G for 15 minutes to obtain the precipitate layer. The precipitate layer was filtered with vacuum suction machine before being condensed by reclaiming solvent by using vacuum evaporator at 45 °C. Dried extract was obtained after removing the rest of moist in condensed extract. Dried extract was powdered and preserved in dessicator.

#### 2.4. Total phenolic compound determination
Evaluation of total phenolic compounds followed the guideline of Hung and Morita [16] with modifications. 0.5 mL of each phenolic sample in various concentration was oxidized with 0.5 mL of Folin–Ciocalteu’s reagent in a centrifuge tube. The reaction was neutralized with 1 mL of saturated sodium carbonate solution, followed by adjusting the volume to 10 mL with distilled water. These components in the tubes were thoroughly mixed and wait for 45 minutes until the blue color developed. Then, these tubes were centrifuged for 5 minutes at 4000G. The absorbance of clear supernatants was measured at wavelength of 725 nm by spectrophotometer (Genesys 10s UV-VIS, Thermo Scientific, USA). Basing on constructed standard curve with ferulic acid, total phenolic content was calculated. The unit was expressed in milligrams of Ferulic acid equivalent (FAE) per gram of sample. Standard calibrations were made in range from 20, 40, 60, 80 and 100 μg/mL. Blank samples were prepared in the same way of extract samples but distilled water was used instead of extract.

2.5. Total flavonoids determination

Flavonoid content of phenolic extracts was determined following the colorimetric method of Chang et al., [17]. 0.5 mL of appropriate dilutions of extracts were mixed with 1.5 mL of 95° ethanol, followed by 0.1 mL of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at wavelength of 415 nm spectrophotometer (Genesys 10s UV-VIS, Thermo Scientific, USA). Basing on constructed standard curve, flavonoid content was calculated. The unit was expressed in micrograms of rutin equivalent (RE) per gram of sample.

2.6. DPPH radical scavenging of phenolic compounds

The scavenging of the stable DPPH radical was widely used to evaluate antioxidant activity of phenolic compounds extracted from fruit and vegetable, cereal grain, wine, etc. In this study, antioxidant activity was evaluated following Blois’s method [18] with modifications. 0.1 mL of each extract sample was mixed with 3.9 mL of 0.075 mM DPPH reagent prepared in methanol. The mixture was allowed to stand for 30 minutes in the dark for enhancing stability. The absorbance of the resulting solution was measured at wavelength of 515 nm. Ascorbic acid of various concentrations (25 μg/ml, 12.5 μg/ml, 6.25 μg/ml, 3.12 μg/ml and 1.56 μg/ml) was used as the standard antioxidant. The control was prepared by adding deionized water to DPPH reagent and the analysis was followed as described above. The results were expressed as percentage inhibition (I %) using the equation below. The IC₅₀ values of the various concentrations were used to build a standard curve using simple linear regression model. Obtained equation in form of \( y = ax + b \) was used for IC₅₀ evaluation.

\[
I\% = \left( \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100.
\]

2.7. Normoglycemic testing

Normoglycemic test was performed following method of Syiem et al. [19] with modifications. Powdered extract from 80° ethanol was used to perform this experiment due to requirement of large amount. Powdered extract was redissolved in distilled water with small amount of ethanol to enhance solubility. 5 groups of 6 non-fasted mice were used in this experiment. 4 tested groups were orally administered extract in dosages of 400 mg/kg b.w, 800 mg/kg b.w, 1600 mg/kg b.w and 4000 mg/kg b.w while control group was given distilled water only. Liquid volume that mice can absorb should not exceed 1mL per 100 gram b.w (OECD
guidelines for the testing of chemicals) [20]. The tested mice were kept under standard condition for 3 months for careful observation. Blood glucose level was recorded by glucometer (Accu-check Performa, USA).

2.8. Glucose tolerance test

Oral-glucose-loading animal model was constructed following method of Syiem et al. [19]. Mice weighing about 25 g were fasted overnight. Pre-tested blood glucose levels of mice level were measured before orally administering glucose solution in dosage of 2 g/kg b.w. After 90 minutes, mice were orally administered extract in 2 dosages of 1600 mg/kg b.w and 4000 mg/kg b.w. Blood glucose levels were recorded at time intervals of 0.5 hours, 1 hour, 2 hours and 24 hours by glucometer (Accu-check Performa, USA). Negative control group was received only saline water while positive control groups were received appropriate dosages of Metformin and Amaryl.

3. RESULTS

3.1 Ethanol extraction efficiency

The amount of total extract depended on the concentration of ethanol used and 96° ethanol solvent showed the lowest efficiency in total extraction while 0° ethanol solvent (distilled water) showed the highest efficiency (Table 1).

Table 1. Crude extract efficiency from various ethanol concentrations.

<table>
<thead>
<tr>
<th>Concentration of ethanol (°)</th>
<th>°96</th>
<th>°90</th>
<th>°80</th>
<th>°70</th>
<th>°50</th>
<th>°25</th>
<th>°0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of crude extract (g/10 g dried powdered)</td>
<td>0.13</td>
<td>1.44</td>
<td>4.79</td>
<td>6.78</td>
<td>8.47</td>
<td>7.18</td>
<td>7.55</td>
</tr>
</tbody>
</table>

3.2. Total flavonoid and total phenolic content

Table 2. Total flavonoid content and Total phenolic content in term of percentage (%)

<table>
<thead>
<tr>
<th>Ethanol Degree</th>
<th>Total flavonoid content* (%)</th>
<th>Total phenolic content** (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>°96</td>
<td>0.404 ± 0.000^d</td>
<td>11.129 ± 1.244^d</td>
</tr>
<tr>
<td>°90</td>
<td>0.113 ± 0.009^b</td>
<td>2.334 ± 0.169^b</td>
</tr>
<tr>
<td>°80</td>
<td>0.053 ± 0.002^c</td>
<td>0.147 ± 0.000^c</td>
</tr>
<tr>
<td>°70</td>
<td>0.041 ± 0.000^d</td>
<td>0.138 ± 0.013^c</td>
</tr>
<tr>
<td>°50</td>
<td>0.014 ± 0.001^c,d</td>
<td>0.087 ± 0.000^d</td>
</tr>
<tr>
<td>°25</td>
<td>0.008 ± 0.002^f,g</td>
<td>0.079 ± 0.009^d</td>
</tr>
<tr>
<td>°0</td>
<td>0.002 ± 0.000^f</td>
<td>0.066 ± 0.009^d</td>
</tr>
</tbody>
</table>

The same final letters (a, b, c, d, e, f, g) in the same column indicate no significant difference.
*Percentage of total flavonoid content in term of g Rutin equivalent (per g crude extract).
** Percentage of total phenolic content in term of g Ferulic acid equivalent (per g crude extract). All values are means of triplicate (P <0.05).
The amount of total flavonoid and phenolic compounds extracted by 96° ethanol was the highest compared to those of other concentrations. Flavonoids-releasing and phenolics-releasing capacities were 0.404 % and 11.129 % respectively (Table 2).

3.3 DPPH radical scavenging

IC\textsubscript{50} value of 90° ethanolic extract was at lowest concentration (8591 µg/ml) while those of 80° ethanolic extract and 70° ethanolic extract were 17791 µg/ml and 19620.69 µg/ml respectively. However, the obtained IC\textsubscript{50} values were still far higher compared to L-ascorbic acid (72.14 µg/ml) (Table 3). Figure 1 shows the changes in inhibition rate of these extracts compared to that of the control.

Table 3. DPPH radical scavenging assay.

<table>
<thead>
<tr>
<th>Radical scavenging agents</th>
<th>Inhibitory Concentration (IC\textsubscript{50}) (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Ascorbic acid</td>
<td>72.14±2.10a</td>
</tr>
<tr>
<td>°70 ethanolic crude extract</td>
<td>19620.69±5.64b</td>
</tr>
<tr>
<td>°80 ethanolic crude extract</td>
<td>17791±6.15c</td>
</tr>
<tr>
<td>°90 ethanolic crude extract</td>
<td>8590.79±7.01d</td>
</tr>
</tbody>
</table>

Concentration of DPPH = 0.075 mM. The same final letters (a, b, c, d) in the same column indicate no significant difference. All values are means of triplicate (P <0.05).

3.4. Normoglycemic test

The results confirm there was no significant difference on blood glucose levels of normoglycemic mice (Table 4).
In vivo study on Ethanolic extract of Allium cepa on mice to evaluate the anti-hyperglycemic activity

Table 4. Normoglycemic test on mice at certain time intervals.

<table>
<thead>
<tr>
<th>Time (Hour)</th>
<th>Dosages (mg/kg b.w)</th>
<th>Control</th>
<th>0.4</th>
<th>0.8</th>
<th>1.6</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>156.83 ± 6.51</td>
<td>163.83 ± 6.46</td>
<td>153.83 ± 3.5</td>
<td>144.67 ± 17.79</td>
<td>152.17 ± 5.71</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>137 ± 11.23</td>
<td>147.17 ± 10.08</td>
<td>135.67 ± 7.47</td>
<td>145 ± 5.06</td>
<td>154 ± 6.54</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>116.5 ± 9.71</td>
<td>117.33 ± 8.45</td>
<td>113.33 ± 9.35</td>
<td>135.83 ± 11.29</td>
<td>114.83 ± 22.86</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>54.83 ± 4.09</td>
<td>70.5 ± 4.96</td>
<td>61.17 ± 4.05</td>
<td>58.67 ± 2.97</td>
<td>69.5 ± 5.34</td>
</tr>
</tbody>
</table>

The same final letters (a, b, c, d) in the same row indicate no significant difference. All values are means of triplicate (P <0.05).

3.5. Glucose tolerance test

Blood glucose level (BGL) after administering dosage of 4 mg/kg b.w decreased slightly after the next 30 minutes but still higher compared to Metformin while dosage of 1.6 mg/kg b.w showed no change. However, after 60 minutes, dosage of 1.6 mg/kg b.w showed significant decline in BGL besides Amaryl. At 60-minute-interval, dosage of 4.0 mg/kg b.w still showed slightly fall while Metformin showed unpredicted upward trend. After 24 hours, BGL of all groups were stable with slight fluctuations that could be ignored (Figure 2).
4. DISCUSSIONS

For safety concern, ethanol was used as solvent for flavonoids and phenolic compounds extraction. Besides the suitable concentration, temperature and soaking time also contribute to extraction efficiency. The result confirms that extraction using 90° ethanol gave highest efficiency with 0.11 g phenolic compounds (gram rutin equivalent) as well as 0.004 g flavonoids (gram ferulic acid equivalent) per one-gram crude extract. Anti-hyperglycemic activity of polyphenol family, especially flavonoid has been proved in many researches. Possessing strong affinity targeting in active site of T2DM-related proteins [21], flavonoid group is considered as one of potential agents for balancing the blood glucose level [22]. The presence of bioactive compounds can control activities of T2DM-related enzyme by tightly binding to their specific sites in order to inhibit their catalytic reaction. The high content of flavonoids and phenolic compounds is also one of key factors for assessment of anti-hyperglycemic and anti-oxidant activity. However, there is a fact that the more polar solvent is used, the more unwanted compounds are uncontrollably extracted. As showed in Table 1, although 96° ethanol can only extract 0.013 g total crude extract per g onion powder, the amount in percentage of phenolic compounds containing flavonoids was the highest (0.404%) compared to the others. As mentioned, 80° ethanolic crude extract was used for experimental approach due to limitation of sufficient amount. Although *A. cepa*’s extract gave no significant effect on blood glucose level of normal mice (Table 4) in normoglycemic test, the extract’s dosage of 4 mg/kg b.w considerably reduced blood glucose level of oral-glucose-loading mice compared to negative control administering saline water and positive control administering Metformin and Amaryl in glucose tolerant test (Figure 2). The anti-hyperglycemic effect of *A. cepa* relates to the presence of extracted compounds. Instant action of crude extract may be explained by multi-target effect, such as insulin-simulating secretion action and rescuing the damaged Beta cells via oxidation processes.

5. CONCLUSION

*In vivo* study demonstrates that ethanolic extract of *A. Cepa* has strong anti-hyperglycemic effect on oral-glucose-loading mice. However, this study only partially reflects a small sight of anti-hyperglycemic activity. For the further studies, the combination of in *in vitro*, *in vivo* and *in silico* study should be carried in order to fully explain both theoretical and experimental aspects. Besides, diabetes-induced mice model is recommended to be used instead of using only non-treated mice. Additionally, extraction method needs to be improved and standardized to enhance the extraction efficiency.

REFERENCES

In vivo study on Ehanolic extract of Allium cepa on mice to evaluate the anti-hyperglycemic activity