Synergistic effects of three traditional herbs green tea, mulberry leaf and corn silk on glucose uptake level of L6 myoblasts and the hypoglycemic mechanism

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Key information

Abstract

Background: Green tea, mulberry leaf and corn silk are traditional herbs used in the prevention and treatment of diabetes in China for a long time, but their synergistic hypoglycemic effects and mechanisms remain unclear. Methods: The effective components of green tea, mulberry leaf and corn silk were extracted and enriched. Mixture design of experiments was used to study the influences of different combinations on the cell viability and glucose uptake level of L6 myoblasts, so as to determine the optimal synergistic hypoglycemic combination. The possible hypoglycemic mechanism of the optimal synergistic combination was explored by cytotoxicity assay, glucose uptake assay, and western blot. Results: Three polyphenol enrichment fractions of the herbs, 30% ethanol elution fraction of green tea (GT), 50% ethanol elution fraction of mulberry leaf (ML) and 60% ethanol elution fraction of corn silk (CS) were obtained. The antioxidant activities of GT-30%, ML-50% and CS-60% were superior to those of crude extracts, and showed strong potential in α-amylase and α-glucosidase inhibition activities. The optimal synergistic combination of crude extracts G7 (crude extract of green tea:crude extract of mulberry leaf:crude extract of corn silk = 1:5:3), polyphenol enrichment fractions R3 (GT-30%:ML-50%:CS-60% = 1:7:1) and monomers X2 (epigallocatechin gallate:boronin:formononetin = 3:1:2) were selected, respectively. G7, R3, and X2 showed promoting effects on the cell viability and glucose uptake of L6 myoblasts within the detected concentration range. In addition, G7, R3, and X2 could increase the expression levels of p-PI3K/PI3K and p-Akt/Akt in L6 myoblasts, and promote the translocation of Glut4, but G7 and R3 showed more significant effects. Conclusion: The synergistic hypoglycemic effects of green tea, mulberry leaf and corn silk had the characteristics of multiple-components and multiple-targets with p-PI3K/PI3K, p-Akt/Akt and the translocation of Glut4 signal pathways involved. The three traditional herbs might have the potential to be combined used for the prevention and treatment of diabetes based on the synergistic hypoglycemic effects.

Keywords: green tea; mulberry leaf; corn silk; synergistic effect; type 2 diabetes mellitus; mechanism

Author contributions

Chen HX conceived and designed the experiments; Zhou JN performed the experiments and drafted the manuscript; Lu JY and Zhang TT contributed in analyzing the data; Lu JY corrected the manuscript; Zhang M and Zhuang PW provided the analysis instrument and the software. All authors read and approved the submission. All authors agreed to be accountable for all aspects of work ensuring integrity and accuracy.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

ABTS, 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; Akt, protein kinase B; CI, combination index; DOE, design of experiments; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DW, dry weight; EGCG, epigallocatechin gallate; FBS, fetal bovine serum; FRAP, ferric reducing antioxidant power; GT, ethanol elution fraction of green tea; ML, ethanol elution fraction of mulberry leaf; CS, ethanol elution fraction of corn silk; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI3K, phosphatidylinositol-3-kinase; SD, standard deviation; T2DM, type 2 diabetes mellitus; TCM, traditional Chinese medicine; 2-NBDG, 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose.

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Highlights
This study found that the main components of the synergistic hypoglycemic effects of green tea, mulberry leaf and corn silk might be polyphenols through the combination of mixture DOE and in vitro cell biology experiments, and three optimal synergistic hypoglycemic combinations were obtained. The hypoglycemic effects of the optimal synergistic hypoglycemic combinations were related to α-amylase inhibition, α-glucosidase inhibition, PI3K/Akt signaling pathway and Glut4 regulation.

Medical history of objective
In "Shennong’s Herbal Classic" (Eastern Han Dynasty), tea is described as having a bitter taste, which can refresh the mind and improve the eyesight after drinking. Modern pharmacology shows that tea can improve insulin activity and insulin resistance, thereby reducing blood sugar levels and alleviating diabetes. Mulberry leaf, first recorded in "Shennong’s Herbal Classic", has the effect of dissipating wind and cooling, clearing lung and moistening dryness (that is to say it can be used for fever and lung heat disease caused by wind-heat cold, dry cough caused by lung dryness, incursion of heat evil, diziness and headache caused by disturbance of upper orifice clearing). Modern pharmacology shows that mulberry leaf can lower blood sugar, blood pressure and blood lipid, etc. The earliest record of the medicinal use of corn silk is "Southern Yunnan Materia Medica" (1476 C.E.), which describes that corn silk has the effect of diuretic detumescence, liver and gallbladder clearing. Modern pharmacology shows that corn silk has many effects such as lowering blood sugar, blood pressure and blood lipid, etc.

Background
Diabetes mellitus is a metabolic disease characterized by hyperglycemia. The common symptoms of diabetes mellitus are polydipsia, polyuria, hyperphagia, and weight loss [1]. There are many types of diabetes mellitus, but type 1 diabetes mellitus and type 2 diabetes mellitus (T2DM) account for the majority of cases. T2DM is more common in patients with diabetes, accounts for about 90% of the total number of diabetes patients [2]. At present, the treatment of T2DM is mainly a combination of diet, exercise, and drug therapy, among which the drug therapy is mainly Western medicine. With the development of traditional Chinese medicine (TCM), the potential of TCM in treating diabetes is gradually being recognized.

In China, the folk application of tea, mulberry leaf and corn silk to prevent and treat diabetes has a long history, and modern pharmacology also shows that all the three have hypoglycemic effects [3-5]. Modern studies confirmed that various active constituents in tea could improve insulin resistance, so as to lower blood glucose level [6]. It has been clear that the hypoglycemic mechanisms of tea mainly include inhibiting the activity of enzymes related to glucose metabolism, improving or protecting the activity of islet cells, inhibiting the activity of transporters related to glucose metabolism, etc. [7-9]. Modern pharmacology also shows that mulberry leaf can directly or indirectly play a hypoglycemic role through a variety of ways. Current studies have confirmed that the hypoglycemic mechanisms of mulberry leaf include promoting insulin release, promoting the utilization of glucose in peripheral tissues, and so on [10]. The hypoglycemic effects of corn silk are mainly reflected in reducing liver glycogen, promoting the utilization of glucose in peripheral tissues, protecting islet cells, and so on [11, 12].

It can be seen that studies on the prevention and treatment of diabetes by tea, mulberry leaf and corn silk have been relatively mature, but most of them are about the hypoglycemic effects of a single component, and their overall effects and specific mechanisms are still unclear. The mechanism of synergetic hypoglycemic effect of tea, mulberry leaf and corn silk is still unclear. In this study, mixture design of experiments (DOE) combined with in vitro experiments was used to explore the possible material basis and mechanism of the synergistic hypoglycemic effect of green tea, mulberry leaf, and corn silk, so as to provide a reference for the development and application of synergistic hypoglycemic medicines or health food.

Materials and methods
Materials
The dried green tea was purchased from Wansuijin Tea Industry (Huanggang, China), the dried mulberry leaf was purchased from Bozhou Shennong Baicaoatang (Bozhou, China), and the dried corn silk was purchased from the local market (Tianjin, China). Epigallocatechin gallate (EGCG, HPLC > 95%) was purchased from Dalian Melun Biological Technology Co., Ltd. (Dalian, China). Morusin (HPLC > 98%) and formononetin (HPLC > 99%) were purchased from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China). D101 macroporous resin and gallic acid (≥ 98% pure) were purchased from Guangfu Fine Chemical Research Institute (Tianjin, China). Ascorbic acid and 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were obtained from Titan Technology Co., Ltd. (Shanghai, China). α-Amylase (3700 U/g) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Solarbio Science & Technology Co., Ltd. (Beijing, China). Acarbose was obtained from Bayer Medical Healthcare Co., Ltd. (Beijing, China). α-Glucosidase (50 U/mg) was purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). L6 skeletal muscle cells were obtained from Fengei Biotechnology Co., Ltd. (Changsha, China). Dulbecco’s modified Eagle medium was purchased from ThermoFisher Biochemical Products Co., Ltd. (Beijing, China). The 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxyglucose (2-NBDG) was purchased from GliBio (Shanghai, China). Metformin was purchased from China Associate Pharmaceutical Co., Ltd. (Shenzhen, China). The primary antibodies including PI3K (19H8), Akt (C57E7), and p-Akt (Ser473) were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). The primary antibody of p-PI3K (ab182651) was purchased from Abcam Trading Co., Ltd. (Shanghai, China). The primary antibody of β-actin (AB0035) was purchased from Abays Biotechnology Co., Ltd. (Shanghai, China). The primary antibody of Glut4 (66846-1-1g) was purchased from Proteintech Group Inc. (Chicago, IL, USA). The secondary antibodies peroxidase-conjugated goat anti-rabbit or mouse IgG were purchased from ZSGB Biotechnology Co., Ltd. (Beijing, China). All other chemicals were of analytical grade.

Methods
Extraction and enrichment of effective components from green tea, mulberry leaf, and corn silk. The 80% ethanol (v/v) was added into green tea powder (1 kg) at the solid-liquid ratio of 1:1.5, then it was extracted by reflux twice (80 °C), for 2 h each time [13]. The 70% ethanol (v/v) was added into mulberry leaf powder (1 kg) according to the solid-liquid ratio of 1:1.5. Reflux extraction (80 °C) was carried out twice for 2 h each time [14]. Corn silk (1 kg) was crushed and added with 80% ethanol (v/v) at a solid-liquid ratio of 1:25, and reflux extraction (70 °C) was performed twice for 2 h each time [15].

The crude extracts of green tea, mulberry leaf, and corn silk were added to the D101 macroporous resin chromatography column, respectively. The crude extracts of green tea and mulberry leaf were eluted with water, 30% ethanol (v/v), 50% ethanol (v/v), 70% ethanol (v/v) and ethanol, respectively. The crude extract of corn silk was eluted with water, 30% ethanol (v/v), 60% ethanol (v/v), 80% ethanol (v/v) and ethanol, respectively. The distilled water elution fractions were discarded. UV-visible colorimetry was used to determine the content of total polyphenols of different fractions according to the previous study [16]. The standard curve was prepared with gallic acid as a standard. The total polyphenols content

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was expressed as mg gallic acid equivalents/g dry weight (DW) of sample.

**Determination of antioxidant activity.** The DPPH radicals scavenging activity, ABTS radicals scavenging activity and ferric reducing antioxidant power (FRAP) of crude extracts and elution fractions of green tea, mulberry leaf and corn silk were analyzed according to the reported methods [17–19]. Ascorbic acid was used as the positive control.

**Determination of inhibitory activities on α-amylase and α-glucosidase.** The α-amylase and α-glucosidase inhibitory activities of crude extracts and elution fractions of green tea, mulberry leaf, and corn silk were analyzed according to the previous studies [20, 21]. Acarbose was used as the positive control.

**Screening of the optimal synergistic hypoglycemic combination of green tea, mulberry leaf, and corn silk.** The culture and differentiation of L6 skeletal muscle cells were performed according to the reported method [22]. According to the results of antioxidant activity, α-amylase inhibitory activity and α-glucosidase inhibitory activity, fractions with the most hypoglycemic potential in green tea, mulberry leaf, and corn silk were selected and combined with different compatibility ratios. In order to better explore the synergistic hypoglycemic mechanism of green tea, mulberry leaf and corn silk, it was decided to include the characteristic polyphenol compounds in three herbs, namely EGCG (characteristic polyphenol of tea), morusin (characteristic polyphenol of mulberry leaf) and formononetin (characteristic polyphenol of corn silk) (Figure 1). The mixture DOE in Minitab 19 software was used for drug combination design. The range of combination proportions was preliminarily screened by MTT assay to remove some of the proportion ranges that had adverse effects on cell survival and growth. The MTT assay method was according to the previous study [22].

The results of MTT assay were input into Minitab 19 software, and quadratic polynomial regression analysis of DOE was carried out to obtain the optimized proportion range. According to the optimized proportion range, different proportions of combinations were designed to study their effects on glucose uptake of L6 myoblasts, so as to select the optimal synergistic combination. The method of glucose uptake by L6 myoblasts was according to the reported study [23]. Metformin (1 mmol/L) was used as the positive control.

**The combination index of the optimal synergistic hypoglycemic combination.** In order to further verify the activity of the optimal synergistic combination, its inhibitory activities of α-amylase and α-glucosidase were studied, and the methods were according to the previous studies [20, 21]. CompuSyn software mainly evaluates the synergistic effect of combination drugs through Chou Talalay model, also known as combination index (CI) equation [24]. Its Equation (1) is:

\[
CI = \frac{x}{\sum_{i=1}^{n} \frac{x_i}{d_i}}
\]

Where, \(\bar{x}\), represented the combination index of a drugs under \(x\%\) inhibition, \(\bar{d_i}\) represented the dose of a single drug that produced a specific effect (such as \(IC_{50}\)), and \(d_i\) represented the dose of each component in the combination drug that produced the same specific effect. When CI < 1, it is synergistic effect (the closer CI value is to 0, the stronger synergy effect is); When CI = 1, it is additive effect, and when CI > 1, it is antagonistic effect [25]. Therefore, the results of α-amylase and α-glucosidase inhibition activity experiments of the optimal synergistic combinations and single components were input into CompuSyn version 1.0 software, so as to judge whether the hypoglycemic effect of the combination was the result of synergistic effect.

**Cytotoxicity of the optimal synergistic hypoglycemic combination.** The cytotoxicity of the optimal synergistic hypoglycemic combination was determined by MTT assay, and the method was according to the previous study [22].

**Effect of the optimal synergistic hypoglycemic combination on glucose uptake in L6 myoblasts.** The 2-NBDG was used to investigate the effect of the optimal synergistic hypoglycemic combination on the glucose uptake level of L6 myoblasts, and the method was according to the reported study [23].

**Western blot of the optimal synergistic hypoglycemic combination.** The total protein, membrane protein, and cytosol protein of L6 myoblasts were extracted according to the previous study [26]. β-Actin was used as the internal reference. The contents of P3ER (1:1,000), p-P3ER (1:500), Akt (1:1,000), p-Akt (1:2,000) and β-actin (1:10,000) in total protein, and Glut4 (1:2,000) and β-actin (1:10,000) in membrane protein and cytosol protein were detected by chemiluminescence and fluorescence imaging system. Then the image was processed by ImageJ software and normalized.

**Statistical analysis.** All measurements were run in triplicate (n = 3). The data was expressed in mean ± standard deviation (SD). The data were analyzed using ANOVA, and \(P < 0.05\) was considered statistically significant. SPSS 20.0 and GraphPad Prism 5 were used for statistical analysis.

**Results**

**Extraction and enrichment of effective components from green tea, mulberry leaf, and corn silk.** After enrichment with D101 macroporous resin, four different fractions of green tea were obtained, which were 30% ethanol elution fraction of green tea (GT), GT-50%, GT-70% and GT-100%. Four different fractions of mulberry leaf were obtained, namely, 30% ethanol elution fraction of mulberry leaf (ML), ML-50%, ML-70% and ML-100%. Four different fractions of corn silk were obtained, namely, 30% ethanol elution fraction of corn silk (CS), CS-60%, CS-80%, and CS-100%. The total polyphenols contents of the crude extracts and elution fractions were determined (Figure 2), and total polyphenols were not detected in GT-100%, ML-100%, and CS-100%. The results showed that GT-30%, ML-50%, and CS-60% were the highest contents of total polyphenols in green tea, mulberry leaf, and corn silk, respectively, and the contents of total polyphenols were 620.50 ± 19.55 mg gallic acid equivalents/g DW of sample, 505.34 ± 5.24 mg gallic acid equivalents/g DW of sample, 509.95 ± 17.93 mg gallic acid equivalents/g DW of sample, respectively. Compared with the crude extracts, the polyphenols of GT-30%, ML-50%, and CS-60% were significantly enriched (\(P < 0.001\)).

**Antioxidant activity**

The crude extracts and elution fractions of green tea, mulberry leaf, and corn silk were tested for the antioxidant activities by DPPH radicals scavenging activity, ABTS radicals scavenging activity and FRAP in vitro assays. The results are shown in Figure 3. The results showed that GT-30% had significant antioxidant activity compared with the other treatments. The order of the antioxidant activities was GT-30% > ML-70% > ML-50% > ML-100% > CS-100% > GT-70% > GT-50% > CS-60% > CS-80% > CS-100% > ML-50% > ML-70% > ML-100% > GT-70% > GT-50% > CS-60% > CS-80% > CS-100%.
with other fractions of green tea (P < 0.05), and the antioxidant activity of GT-30% was similar to that of the positive control. Among the fractions of mulberry leaf, ML-30% and ML-50% showed significant antioxidant activities compared with other fractions (P < 0.05). Among the fractions of corn silk, CS-30% and CS-60% had significant antioxidant activity compared with other fractions (P < 0.05). It could be seen that the fractions with high polyphenol content had stronger antioxidant activity than the fractions with low polyphenol content, which indicated that polyphenols might be the main antioxidant constituents in green tea, mulberry leaf, and corn silk. In addition, among the three herbs, green tea had the strongest antioxidant activity.

**Inhibitory activities of α-amylase and α-glucosidase**

The α-amylase and α-glucosidase inhibitory activities of crude extracts and elution fractions of green tea, mulberry leaf, and corn silk were determined in vitro, and the IC₅₀ values were shown in Table 1. The results showed that the inhibitory activities of α-amylase and α-glucosidase in high polyphenol content fractions of green tea and corn silk (GT-30%, GT-50%, and CS-60%) were stronger than those in low polyphenol content fractions. The α-amylase inhibitory activity of high polyphenol content fractions of mulberry leaf (ML-30% and ML-50%) was strong, but the α-glucosidase inhibitory activity was not as strong as that of low polyphenol content fractions. After enrichment, GT-30% and CS-60% showed stronger enzyme inhibitory activities than crude extracts. Therefore, the polyphenols in green tea and corn silk might be the main active constituents to inhibit α-amylase and α-glucosidase.
Screening of the optimal synergistic hypoglycemic combination of green tea, mulberry leaf, and corn silk

Considering the results of antioxidant activity and enzyme inhibition activity, polyphenol enrichments of green tea, mulberry leaf, and corn silk were selected for further synergistic hypoglycemic study. In addition, the synergistic hypoglycemic combination of crude extracts and monomers of green tea, mulberry leaf, and corn silk were also studied, so as to discover the synergistic mechanism in the comparison.

The fixed total concentration of crude extracts combination and polyphenol enrichments combination was 90 μg/mL, and only the different proportions of the three components in the combination were changed, so as to obtain the combination schemes (Table 2, 3) by Minitab 19 software. Similarly, the total fixed concentration of the monomers combination was 30 μmol/L, and only the different proportions of the three compounds in the combination were changed (Table 4).

According to Table 2-4, samples of different proportions were prepared and MTT assay was carried out. The MTT results (Figure 4) were input into Minitab 19 software for analysis, and the results were obtained as shown in Figure 5.

Table 1 The IC50 values of crude extracts and elution fractions of green tea, mulberry leaf and corn silk about the α-amylase and α-glucosidase inhibitory activities

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample</th>
<th>IC50 of α-amylase (mg/mL)</th>
<th>IC50 of α-glucosidase (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>Acarbose</td>
<td>0.079 ± 0.003a</td>
<td>0.004 ± 0.001a</td>
</tr>
<tr>
<td></td>
<td>Crude extract</td>
<td>2.147 ± 0.128a</td>
<td>29.059 ± 3.076c</td>
</tr>
<tr>
<td></td>
<td>GT-30%</td>
<td>0.524 ± 0.091a</td>
<td>2.328 ± 0.241b</td>
</tr>
<tr>
<td>Green tea</td>
<td>GT-50%</td>
<td>0.471 ± 0.031a</td>
<td>2.108 ± 0.165a</td>
</tr>
<tr>
<td></td>
<td>GT-70%</td>
<td>28.662 ± 2.829d</td>
<td>13.235 ± 1.217f</td>
</tr>
<tr>
<td></td>
<td>GT-100%</td>
<td>12.709 ± 0.565e</td>
<td>57.292 ± 2.809d</td>
</tr>
<tr>
<td></td>
<td>Crude extract</td>
<td>1.950 ± 0.188a</td>
<td>53.318 ± 6.122b</td>
</tr>
<tr>
<td></td>
<td>ML-30%</td>
<td>0.226 ± 0.002a</td>
<td>182.193 ± 12.020c</td>
</tr>
<tr>
<td>Mulberry leaf</td>
<td>ML-50%</td>
<td>0.331 ± 0.019a</td>
<td>64.061 ± 13.781b</td>
</tr>
<tr>
<td></td>
<td>ML-70%</td>
<td>11.451 ± 0.322c</td>
<td>11.456 ± 0.710c</td>
</tr>
<tr>
<td></td>
<td>ML-100%</td>
<td>14.044 ± 0.187c</td>
<td>18.328 ± 1.358e</td>
</tr>
<tr>
<td></td>
<td>Crude extract</td>
<td>1.199 ± 0.046a</td>
<td>101.952 ± 8.476c</td>
</tr>
<tr>
<td></td>
<td>CS-30%</td>
<td>0.659 ± 0.044a</td>
<td>152.105 ± 9.496d</td>
</tr>
<tr>
<td>Corn silk</td>
<td>CS-60%</td>
<td>0.308 ± 0.041a</td>
<td>26.431 ± 3.400d</td>
</tr>
<tr>
<td></td>
<td>CS-80%</td>
<td>10.480 ± 1.066b</td>
<td>10.819 ± 2.087b</td>
</tr>
<tr>
<td></td>
<td>CS-100%</td>
<td>11.572 ± 0.449e</td>
<td>101.551 ± 4.558c</td>
</tr>
</tbody>
</table>

Different letters (a–d) indicated significant differences within the same group (P < 0.05). All measurements were run in triplicate (n = 3). The data was expressed as mean ± SD; GT, ethanol elution fraction of green tea; ML, ethanol elution fraction of mulberry leaf; CS, ethanol elution fraction of corn silk.

Table 2 The combination scheme of crude extracts of green tea, mulberry leaf and corn silk

<table>
<thead>
<tr>
<th>Combination number</th>
<th>Crude extract of green tea (μg/mL)</th>
<th>Crude extract of mulberry leaf (μg/mL)</th>
<th>Crude extract of corn silk (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>90</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>b</td>
<td>60</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>c1</td>
<td>45</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>c2</td>
<td>45</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>d</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>e1</td>
<td>15</td>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td>e2</td>
<td>15</td>
<td>15</td>
<td>60</td>
</tr>
<tr>
<td>f1</td>
<td>0</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>f2</td>
<td>0</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>f3</td>
<td>0</td>
<td>0</td>
<td>90</td>
</tr>
</tbody>
</table>

Table 3 The combination scheme of polyphenol enrichments of green tea, mulberry leaf and corn silk

<table>
<thead>
<tr>
<th>Combination number</th>
<th>GT-30% (μg/mL)</th>
<th>ML-50% (μg/mL)</th>
<th>CS-60% (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>g</td>
<td>90</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>h</td>
<td>60</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>i1</td>
<td>45</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>i2</td>
<td>45</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>j</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>k1</td>
<td>15</td>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td>k2</td>
<td>15</td>
<td>15</td>
<td>60</td>
</tr>
<tr>
<td>l1</td>
<td>0</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>l2</td>
<td>0</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>l3</td>
<td>0</td>
<td>0</td>
<td>90</td>
</tr>
</tbody>
</table>

GT, ethanol elution fraction of green tea; ML, ethanol elution fraction of mulberry leaf; CS, ethanol elution fraction of corn silk.
Table 4 The combination scheme of monomers of green tea, mulberry leaf and corn silk

<table>
<thead>
<tr>
<th>Combination number</th>
<th>EGCG (μmol/L)</th>
<th>Morusin (μmol/L)</th>
<th>Formononetin (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>m1</td>
<td>0</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>m2</td>
<td>0</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>m3</td>
<td>0</td>
<td>15</td>
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<tr>
<td>n1</td>
<td>5</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>n2</td>
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EGCG, epigallocatechin gallate.

Figure 4 The cytotoxicity of the different combinations on L6 myoblasts. (A) Crude extracts. (B) Polyphenol enrichments. (C) Monomers. All measurements were run in triplicate (n = 3). The data was expressed as mean ± SD. Compared with control, *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 5 Mixed contour maps of different combinations on cell viability (A, crude extracts; C, polyphenol enrichments; E, monomers) and surface diagram of different combinations on cell viability (B, crude extracts; D, polyphenol enrichments; F, monomers). GT, ethanol elution fraction of green tea; ML, ethanol elution fraction of mulberry leaf; CS, ethanol elution fraction of corn silk.
Setting the appropriate limit value can control the subsequent verification range within a reasonable range. Therefore, under the condition that the fixed total concentration remained unchanged at 90 μg/mL, the proportion range after the optimization of the synergistic combination of crude extracts was: X axis (crude extract of GT): 0 to 40, Y axis (crude extract of ML): 50 to 90, Z axis (crude extract of CS): 0 to 34. Under the condition that the fixed total concentration remained unchanged at 90 μg/mL, there were two optimized proportion ranges of the synergistic combination of polyphenol enrichments. One was: X axis (GT-30%): 0 to 38, Y axis (ML-50%): 50 to 90, Z axis (CS-60%): 0 to 8. The other was: X axis (GT-30%): 23 to 49, Y axis (ML-50%): 0 to 5, Z axis (CS-60%): 39 to 66. Under the condition that the fixed total concentration remained unchanged at 30 μmol/L, the proportion range after the optimization of the synergistic combination of monomers was: X axis (EGCG): 0 to 30, Y axis (morusin): 0 to 6, Z axis (formononetin): 0 to 30.

According to the optimized proportion ranges, different proportion combinations were designed for 2-NBDG experiment. The total concentrations of the crude extract combination and polyphenols enrichment combination were fixed at 90 μg/mL, and only the different proportions of the three components in the combination were changed (Supplementary Table S1, S2). The fixed total concentration of monomer combination was 30 μmol/L, only the different proportions of the three monomers in the combination were changed (Supplementary Table S3). The results were shown in Figure 6. The results showed that for the combination of crude extracts, when the crude extract of green tea: the crude extract of mulberry leaf: the crude extract of corn silk = 1:5:3, that was, G7 group, L6 myoblasts had the highest 2-NBDG uptake rate (P < 0.001). Therefore, G7 was the optimal synergistic combination of crude extracts. For the polyphenol enrichment combinations, when GT-30%:ML-50%:CS-60% = 1:7:1, that was, R3 group, L6 myoblasts had the highest 2-NBDG uptake rate (P < 0.001). Therefore, R3 was the optimal synergistic combination of polyphenol enrichments. For the combination of monomers, when EGCG:morusin:formononetin = 3:1:2, that was, X2 group, L6 myoblasts had the highest 2-NBDG uptake rate (P < 0.001), so X2 was the optimal synergistic combination of monomers.

**The combination index of the optimal synergistic hypoglycemic combination**

In order to further verify the activity of the optimal synergistic combination, the inhibitory activities of G7, R3, and X2 on α-amylase and α-glucosidase were studied and compared with those of single components (Table 5). The results showed that the α-amylase and α-glucosidase inhibitory activities of G7 and X2 were stronger than those of single components. The α-amylase inhibitory activity of R3 was stronger than that of single components, but its α-glucosidase inhibitory activity was not as strong as that of GT-30%.

![Figure 6 The 2-NBDG uptake of the different combinations on L6 myoblasts.](https://www.tmrjournals.com/tmr)
Western blot of the optimal synergistic hypoglycemic combination

The concentration corresponding to the highest 2-NBDG uptake level of each sample was selected for western blot assay. Therefore, the concentrations of G7 and R3 were 200 μg/mL, and the concentration of X2 was 80 μg/mL. The positive group was metformin (1 mmol/L) and the control group was Dulbecco’s modified Eagle medium. The effects of each sample on P3IK, p-P3IK, Akt, p-Akt, and Glut4 were shown in Figure 8C–8E. Among them, the expression levels of P3IK, p-P3IK, Akt, and p-Akt could reflect the influence of each sample on P3IK/Akt pathway. The results showed that compared with the control group, G7 had significant effects on the activation of P3IK/Akt pathway and the translocation of Glut4 (P < 0.05). The effect of R3 on the activation of P3IK/Akt pathway and the translocation of Glut4 was similar to that of metformin. X2 could promote the activation of P3IK/Akt pathway and the translocation of Glut4, but had no significant effect. These results suggested that G7 and R3 might promote glucose uptake of L6 myoblasts by regulating the P3IK/Akt signaling pathway and Glut4, thus achieving the purpose of anti-diabetes. However, the hypoglycemic effect of X2 in L6 myoblasts might not be mainly dependent on the P3IK/Akt signaling pathway and Glut4, but other pathways played a major role.

Discussion

Diabetes is a metabolic disease characterized by hyperglycemia. More and more studies showed that oxidative stress was related to the pathogenesis of T2DM and its complications. Metabolic disorder could lead to oxidative stress and damage the antioxidant defense system of T2DM patients [27–29]. Therefore, it is possible to study whether medicines have the potential of anti-diabetes through antioxidant activity experiments. Generally, antioxidant activity can be manifested by FRAP assay and scavenging radicals, including DPPH radicals and ABTS radicals. The results showed that fractions with higher polyphenol content had stronger advantages in antioxidant activity. Experimental designs showed that plant polyphenols were effective natural antioxidants, and their antioxidant activity was closely related to their phenolic structure. In addition, the properties of substituents, degree of polymerization, and degree of glycosylation also affected the antioxidant activity of plant polyphenols [30, 31]. Therefore, the polyphenol enrichments of green tea, mulberry leaf, and corn silk showed different antioxidant activities, which might be mainly due to the different structural characteristics of polyphenol compounds in the three, which remained to be further studied. However, in general, the polyphenol enrichments of the three had good antioxidant activity and might have certain hypoglycemic potential.
Figure 7 The CI plot of the optimal synergistic combinations on inhibitory activity of α-amylase (A, crude extracts; C, polyphenol enrichments; E, monomers) and CI plot of the optimal synergistic combinations on inhibitory activity of α-glucosidase (B, crude extracts; D, polyphenol enrichments; F, monomers).

Figure 8 The cytotoxicity (A) and 2-NBDG uptake (B) of the optimal synergistic combinations on L6 myoblasts. The effect of the optimal synergistic combination on PI3K/Akt pathway (C, D) and translocation of Glut4 (E) in L6 myoblasts. All measurements were run in triplicate (n = 3). The data was expressed as mean ± SD. Compared with control, *P < 0.01; **P < 0.001. Different letters (a, b) indicated significant differences (P < 0.05). 2-NBDG, 2-{N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino}-2-deoxyglucose.
To further investigate whether polyphenol enrichments of green tea, mulberry leaf, and corn silk have the potential of hypoglycemic effect, the inhibitory activities of α-amylase and α-glucosidase were measured. α-Amylase and α-glucosidase are two key enzymes for human body to digest and absorb carbohydrates. α-Amylase can hydrolyze the α-1,4-glucoside bond, cutting the starch chain into short-chain oligosaccharides, and α-glucosidase can hydrolyze the glucoside bond in the molecular chain of oligosaccharides, so as to release absorbable glucose [32]. They are directly involved in the metabolism of starch and glycogen. Therefore, by inhibiting the activities of α-amylase and α-glucosidase, the glucose release can be reduced and the postprandial blood glucose level can be effectively reduced, which reflects the anti-diabetic ability of medicines to a certain extent [33]. The α-amylase and α-glucosidase inhibitory activities of polyphenol enrichments in green tea and corn silk were stronger than those of crude extracts. However, it could be seen that the α-amylase inhibitory activity of ML-50% was strong, while its α-glucosidase inhibitory activity was not so strong. This result might be caused by the fact that the main parts of polyphenols in mulberry leaf to play a hypoglycemic role were in the oral cavity and gastrointestinal saliva, while the main parts of other compounds to play a hypoglycemic role were in the intestinal mucosa, which needs to be further researched. In addition, it could be found that in terms of α-amylase inhibitory activity, among the three polyphenol enrichments, CS-60% showed the strongest activity, followed by ML-50%, and GT-30% showed the weakest activity. In terms of α-glucosidase inhibitory activity, among the three polyphenol enrichments, GT-30% had the strongest activity, followed by CS-60%, and ML-50% showed the weakest activity. These results indicated that there were differences in enzyme inhibitory activities among green tea, mulberry leaf, and corn silk, so they might have synergistic hypoglycemic effects by acting on different sites or pathways when used in combination. In a word, polyphenol enrichments of green tea, mulberry leaf, and corn silk had the potential of hypoglycemic effect.

Drug synergy, in short, is to achieve the effect of “1 + 1 > 2”. This kind of synergy is not just a simple superposition of efficacy but an interaction mode of action, promoting strengths and avoiding weaknesses, with advantages of multiple targets and multiple pathways, and can enhance activity, reduce dose, and reduce toxicity. At present, the application and research of green tea, mulberry leaf, and corn silk in the prevention and treatment of diabetes are very extensive, and the established mechanisms include inhibiting the activity of enzymes related to glucose metabolism, promoting the utilization of glucose by peripheral tissues, and protecting islet cells, etc. [5, 7]. However, there is currently limited research on the synergistic hypoglycemic effect of green tea, mulberry leaf, and corn silk, and the mechanism of their synergistic hypoglycemic effect is still unclear.

Carbohydrate digestion by enzymes is eventually absorbed by intestinal epithelial cells in the form of glucose, and glucose uptake and utilization are mainly found in peripheral tissues or cells, such as liver, muscle, and adipose cells [34]. Therefore, the ability of a drug to promote glucose uptake by peripheral cells is the true reflection of its hypoglycemic ability. Skeletal muscle, as one of the largest organs in normal human body, is responsible for up to 80% of postprandial glucose uptake [35]. In insulin-resistant states, such as T2DM and obesity, insulin-stimulated glucose disposal in skeletal muscle is markedly impaired [36]. In this case, skeletal muscle is an important tissue for peripheral cells to uptake glucose, and plays an important role in muscle maintenance of blood glucose homeostasis. Meanwhile, impaired skeletal muscle glucose uptake and utilization play a key role in the development and progression of T2DM. Insulin resistance in skeletal muscle is a precursor in the development of T2DM [36]. Many works have focused on the glucose uptake of skeletal muscle cells to explore the potential on the T2DM treatment [37–39]. Therefore, in this study, the optimal synergistic hypoglycemic effect of crude extracts (GT, crude extract of green tea crude extract of mulberry leaf, crude extract of corn silk = 1:5:3), the optimal synergistic hypoglycemic combination of polyphenol enrichments (R, GT-30%M: ML-50%: CS-60% = 1:7:1) and the optimal synergistic hypoglycemic combination of monomers (X2, EGCG: morusin: formononetin = 3:1:2) were obtained by combining mixture DOE and L6 myoblast model.

G7, R3, and X2 showed no obvious cytotoxicity to L6 myoblasts. Further experiments on glucose uptake in L6 myoblasts showed that G7 and R3 could significantly promote glucose uptake within a certain concentration molecular range, while X2 had no significant effect on promoting glucose uptake in L6 myoblasts. The results of CI analysis showed that G7 and R3 had strong synergistic effects on α-amylase and α-glucosidase inhibitory activities, while X2 showed strong synergistic effect on α-amylase inhibitory activity, but had antagonistic effect on α-glucosidase inhibitory activity. A large number of studies showed that P38K/Akt signaling pathway was closely related to the occurrence and development of T2DM, and the activation of PI3K/Akt signaling pathway was mainly through the phosphorylation of P38K and Akt [40, 41]. In addition, insulin regulated Glut4 membrane transport was crucial for maintaining the body’s blood glucose balance, as Glut4 performs glucose transport functions on the cell membrane [42, 43]. G7, R3, and X2 all could promote the expression of P38K/Akt pathway and the translocation of Glut4, but G7 and R3 had more significant effects. These above results indicated that the synergistic hypoglycemic effect of green tea, mulberry leaf and corn silk might be not the result of interaction of several single compounds, but the result of multi-compounds interaction, and polyphenols might play an important role in the process of multi-compounds interaction. Moreover, the synergistic hypoglycemic mechanism of green tea, mulberry leaf, and corn silk was related to the regulation of P38K/Akt pathway and Glut4.

In this study, modern analytical methods were used to confirm the synergistic hypoglycemic potential of green tea, mulberry leaf, and corn silk and their possible mechanism. Based on the proportion of the three optimal synergistic hypoglycemic combinations obtained, mulberry leaf was more likely to be the principal medicine. At the same time, considering that both green tea and mulberry leaf were cold-property medicines, the addition of corn silk (neutral-property medicine) could neutralize some cold properties, which was also a protective effect on the body. Therefore, the three combinations obtained in this study had the potential to prevent diabetes and alleviate the initial symptoms of diabetes.

Conclusions

This study enriched the bioactive fractions, firstly investigated the material basis and potential synergistic hypoglycemic mechanism of green tea, mulberry leaf, and corn silk. It was found that the main components of the synergistic hypoglycemic effect of green tea, mulberry leaf, and corn silk might be multiple polyphenols through the combination of mixture DOE and in vitro cell biology experiments, and the three optimal synergistic hypoglycemic combinations were obtained. The hypoglycemic effects of the optimal synergistic hypoglycemic combinations were related to α-amylase inhibition, α-glucosidase inhibition, P38K/Akt signaling pathway, and Glut4 regulation. The synergistic hypoglycemic effects of green tea, mulberry leaf, and corn silk had the characteristics of multiple-components and multiple-targets with p-P38K/P38K, p-Akt/Akt and the translocation of Glut4 signal pathways involved. The three traditional herbs might be combined used for the prevention and treatment of diabetes based on the synergistic hypoglycemic effects. This study laid a foundation for the research and development of related synergistic hypoglycemic medicines and functional food.

References

2. American Diabetes Association. Diagnosis and classification of


