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Abstract

Background: The seeds of *Toona sinensis* (Juss.) M. Roem (*T. sinensis*) have long been used in Traditional Chinese Medicine for the treatment of diabetes mellitus (DM) and its complications. The aim of this study was to investigate the antioxidant activity of different polyphenols fractions from *Toona sinensis* Roem (*T. sinensis*) seeds (PTSS) and the inhibition of aldose reductase (AR).

Methods: Macroporous resin was used to purify PTSS, and the antioxidant activities were evaluated with total antioxidant capacity and free-radical scavenging. AR inhibitory activities were investigated by employing various established systems.

Results: The polyphenol eluted by 60% alcohol (PTSS3) exhibit the highest antioxidant activity and AR inhibition, with an r value of 0.9924 ± 0.0066 in correlation analysis. The inhibition mechanism of PTSS3 on AR is uncompetitive inhibition.

Conclusion: This research demonstrates that PTSS offers potential for intervening diabetes mellitus and its complications.

Key words: *Toona sinensis* seeds, polyphenol, antioxidization, aldose reductase, diabetes mellitus

Introduction

Diabetes mellitus (DM) is a common and frequently occurring disease. In DM patients, long-term hyperglycemia damages the main vessels and blood capillaries and endangers the heart, brain, kidney, peripheral nerves, eyes, and feet, thereby significantly threatening the health and lives of diabetics. DM complications are related to the polyol pathway (PP) which contributes to glucose metabolism (Jang et al., 2010; Manna et al., 2009), meanwhile aldose reductase (AR) is the key rate-limiting enzyme of PP (Schemmel et al., 2010; Alexiou et al., 2009; Srivastava et al., 2005); that is, AR inhibitors (ARI) can inhibit AR activity and interfere PP metabolism and thus interfere the initiation and progression of DM complications. In addition, according to the unified mechanism theory of DM complications, oxidative stress is an independent factor of DM and its complications activate the pathological pathways that are related to nearly all DM complications, thereby becoming the key link of DM pathogenesis (Feng et al., 2013; Kumar & Kar, 2015; Kassab & Piwowar, 2012).

Plant polyphenol is a polyhydroxy phenolic compound that widely exists in plants. The unique structure of polyphenol grants its many distinctive physiological functions like anti-tumor, anti-oxidization, anti-arteriosclerosis, and antiseptic properties and prevention of coronary heart disease, stroke, and other cardiovascular diseases (Amico et al., 2008; Al-Awwadi et al., 2004). As the research on natural products attracts increasing attention, plant polyphenols have gradually become a research focus.

Toona sinensis (Juss.) M. Roem (*T. sinensis*) is extensively distributed in China as a medicinal and edible plant with significant utilization value. It possesses antiseptic, anti-inflammatory, analgesic, anti-cancer and antioxidant activities, and can inhibit platelet aggregation and lower blood glucose levels (Liu et al., 2014; Yang et al., 2011; Yu et al., 2012). Chemical compositions of *T. sinensis* seeds include polyphenol, saponin, sesquiterpene, alkaloid, and volatile oil (Kakumu et al., 2014; Mu et al., 2007). Based on preliminary work, this research investigated the antioxidant and ARI activities of *T. sinensis* seeds and preliminarily screened the active *T. sinensis* seeds' polyphenol constituents, in the hope of finding their potential prevention to DM complications.

Materials and Methods

Instruments and Drugs

The materials used in this study are the following: *T. sinensis* seeds (purchased from Jinan Shengke Technology Development Co., Ltd. China, and identified to be seeds of *T. sinensis* (A. Juss.) Roem by Dr. Xu Chongmei of the Department of Pharmacognosy of Weifang Medical University); New Zealand white rabbits (provided by Weifang Medical Experimental Animal Center.); HPD400 macroporous resin (from Cangzhou Bon Adsorber Technology Co., Ltd. China.); BioTek microplate reader (Gene Co., Ltd, USA.); HC-2518R high-speed refrigerated centrifuge (Anhui Zhongke Zhongjia Scientific Instruments Co., Ltd. China.); 10-585 ultrasonic cell grinder (Ningbo Xinzhi Biological Technology Co., Ltd. China.); UV8000 UV-visible spectrophotometer (Shanghai Precision Instrument Co., Ltd. China.); Epalrestat (standard, the content > 99.4%, National Institutes for Food and Drug Control, China.); reduced coenzyme II/DL-glyceraldehyde (Shanghai Yuanye Bio-technology Co., Ltd, China.); and total antioxidant capability and superoxide anion kits (batch numbers 20130726 and 20130806, respectively, Nanjing Jiancheng Bioengineering Institute, China). All experiments on animals were conducted in accordance with and after approval by the Institution Animal Ethics Committees.

Extraction and Purification of Polyphenol from *T. Sinensis* Seeds

Approximately 250 g of *T. sinensis* seeds were grinded and then degreased twice with 800 mL of ether. When the ether in the residues had volatilized, acetone-water (1:1) that is, twice the volume of the residues were added for extraction. The process was repeated twice, and each lasted

for 3 h. The filtrate was combined, condensed, and then freeze-dried. Finally, a brown powder was obtained and marked as the polyphenols from *T. sinensis* seeds (hereafter referred to as PTSS).

Pure water was added to PTSS, and the mixture was sonicated. The final concentration was 0.15 mg/mL. The sample was loaded into the HPD400 macroporous resin column and then eluted with 20%, 40%, and 60% alcohol, separately; the eluents were collected, condensed, and freeze-dried to obtain the purified components of PTSS, which were marked as PTSS1, PTSS2, and PTSS3, individually. Qualitative detection of polyphenolic components were performed on these extracts using ferrous tartrate solution, and the results show positive reaction.

Detection of Polyphenol Contents

Folin-phenol colorimetry detected the content of PTSS of different parts (Subedi et al., 2014). Different volumes (0, 20, 50, 100, 150, 200, and 250 μ L) of normal gallic acid solution at 0.2 mg/mL were precisely measured and added to water to reach a final volume of 250 μ L. Exactly 0.4 mL of folin-phenol reagent was added into the solution, which was then mixed and placed statically for 1 min. Sodium carbonate solution (0.4 mL, 10%) and distilled water (4.0 mL) were then added, and the mixture was shaken. After 20 min of color development, the absorbance of the tubes was detected at 760 nm wavelength. With the content of gallic acid control as horizontal coordinate and the absorbance as vertical coordinate, the standard curve was drawn.

Polyphenol extracts (100 μ L, 3.0 mg/mL) were detected with the method mentioned above and the total polyphenol content in the extracts was calculated (%).

Determination of PTSS Antioxidant Activity: Determination of Total Antioxidant Capability

The total antioxidant capability kit (T-AOC) and microplates were used for quantitative assay. The reaction principle is based on the reduction of Fe^{3+} to Fe^{2+} and Fe^{2+} together with Fehling's substances from stable complex compounds. The polyphenol extracts were dissolved with 60% alcohol; with the concentration (mg/mL) as horizontal coordinate and the absorbance as vertical coordinate, a reducing power curve was drawn. Ascorbic acid was used for positive control.

Determination of DPPH Radical Scavenging

DPPH was first dissolved with a small amount of absolute alcohol (Fu et al., 2013) and then diluted with 60% alcohol to be 0.2 mmol/L. The polyphenol extracts were dissolved with 60% alcohol at different concentrations. During the reaction, the same volume of DPPH solution was added, and the solution was placed away from light for 15 min. The absorbance was detected immediately at 515 nm wavelength with ascorbic acid as positive control.

Determination of PTSS' Capability of Scavenging Superoxide Anion and Hydroxyl Radical

The anti-superoxide anion and hydroxyl radical kits were used with ascorbic acid as standard to determine the PTSS' capability of scavenging reactive oxygen radicals.

Inhibitory Effect of PTSS on AR: Preparation of AR

The rabbits were anesthetized and the eyeball lenses were separated. Two lenses were placed into a centrifuge tube and cut into pieces, and the 1 mL of pre-cooled distilled water was added. In an ice bath, they were sonicated from an opaque white lens suspension, and then centrifuged at 4 °C (17465 g \times 30 min). The colorless and transparent homogenate lens supernatant was taken and placed at 4 °C for usage within 24 h.

Determination of the Inhibitory Effect of PTSS on AR

The reaction was conducted in a 96-well ELISA plate, and the total volume was 100 μ L per well. The sample was added according to the volumes listed in Table 1, in triplicate with epalrestat as positive control drug. After adding coenzyme NADPH (0.16 mmol/L) and substrate DL-glyceraldehyde (10 mmol/L), the microplate reader monitored the absorbance of NADPH at 340 nm for successive 20 min. The capability of inhibiting coenzyme metabolism after the reaction of enzyme and substrate was used to express enzyme activity. The enzyme activity at 60% to 70% time point was taken to calculate the samples' AR inhibition rate. To ensure the consistency of the results, the detection of enzyme activity is required in each experiment to determine the appropriate reaction time. The inhibition curve was drawn with sample concentration as horizontal coordinate and inhibition rate as vertical coordinate.

Table 1: Reaction system of AR determination

	buffer (μ L)	enzyme solution (μ L)	drug (μ L)	NADPH (μ L)	NADPH- substrate (μ L)
blank group	90	10	-	-	-
standard group	10	10	-	80	-
control group	10	10	-	-	80
drug group	0	10	10	-	80
blank drug group	80	10	10	-	-

The formulas used are as follows:

Enzyme activity (%) = $[(A_{\text{standard}} - A_{\text{control}}) / (A_{\text{standard}} - A_{\text{blank}})] \times 100\%$

Inhibition rate (%) = $\{[(A_{\text{drug}} - A_{\text{blank drug}}) - (A_{\text{control}} - A_{\text{blank}})] / (A_{\text{standard}} - A_{\text{control}})\} \times 100\%$

Determination of PTSS' Inhibition Type on AR

The eluting fragment of PTSS with marked inhibition were selected to investigate the inhibition types of AR. Based on the method above, blank group and standard group can be excluded, and the concentrations of the substrate were 0.067, 0.1, 0.2, 0.5, and 1.0 mmol/L. At 0.05 and 0.1 mg/mL concentrations, a decrease in NADPH absorbance was recorded at every minute. Double-reciprocal plot was used to determine the type of inhibition. The kinetic constants were calculated based on Michaelis-Menten equation (Kumar et al., 2011).

Correlation Analysis of Antioxidant Activity and AR Inhibition

Linear regression was conducted between total antioxidant activity and inhibition of AR activity to evaluate the correlation of oxidative stress and the AR inhibition.

Statistical Analysis

The experimental results were subjected to analysis of variance using SPSS 16.0 and expressed as mean \pm SD.

Results

Determination of Polyphenol Contents

Based on folin-phenol method, the standard curve of gallic acid was determined to be $y = 19.18x - 0.0042$ ($r = 0.9994$), whereas the polyphenol content in PTSS, PTSS1, PTSS2, and PTSS3 were $4.62\% \pm 0.24\%$, $4.31\% \pm 0.21\%$, $14.10\% \pm 0.18\%$, and $11.33\% \pm 0.28\%$ respectively.

Antioxidant Activity of PTSS

The results in Fig. 1 show that PTSS3 in the PTSS extracts has the greatest antioxidant activity, followed by PTSS2, PTSS, and PTSS1. Although the polyphenols from *T. sinensis* seeds have a total antioxidant capability inferior to ascorbic acid, they can still provide a natural material for efficiently separating and purifying natural antioxidant components.

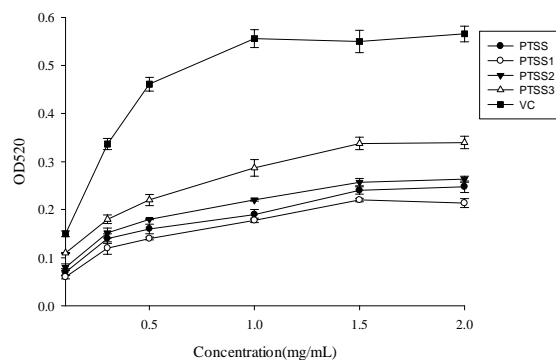


Figure 1: Determination results of the T-AOC of the polyphenols from *T. sinensis* seeds

The IC_{50} values of PTSS and ascorbic acid (positive control drug) on three reactive oxygen radicals were detected and the results are listed in Table 2. Table 2 indicates that PTSS can well scavenge DPPH and O_2^- , and the inhibition is ordered as $PTSS3 > PTSS2 > PTSS > PTSS1$. Hence, PTSS is advantageous in scavenging DPPH radicals.

Table 2: IC_{50} of polyphenols on reactive oxygen radicals ($\mu\text{g/mL}$, mean \pm SD, $n = 5$)

	PTSS 1	PTSS 2	PTSS 3	PTSS	ascorbic acid
DPPH	30.15 ± 0.93	14.45 ± 1.27^a	10.17 ± 1.29^b	20.25 ± 1.36^{bc}	5.18 ± 0.75
O_2^-	103.98 ± 2.94	94.55 ± 2.23^a	68.18 ± 2.19^b	96.96 ± 2.77^{bc}	35.32 ± 1.58
$HO\cdot$	277.69 ± 19.88	219.29 ± 12.31^a	189.33 ± 9.67^b	230.01 ± 15.14^{bc}	71.13 ± 5.09

^a $P < 0.001$ when compared with PTSS3; ^b $P < 0.001$ when compared with PTSS2; ^c $P < 0.001$ when compared with PTSS1

Inhibitory Effect of PTSS on AR

The enzyme activity changed with time and the enzyme activity could reach 60%-70% in approximately 5 min. Fig. 2 shows the inhibition curve of PTSS on AR. The results indicate that PTSS inhibits AR at varied degrees, and the performance of PTSS3 is remarkable, which is significantly better than that of PTSS.

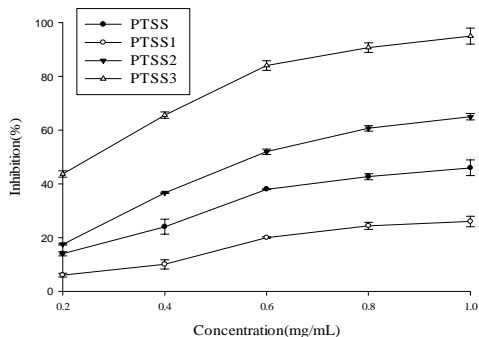


Figure 2: Inhibitory effect of the polyphenols on AR

Epalrestat is the only marketed drug as an AR inhibitor, and its AR inhibition rates at different concentrations are listed in Table 3. As the concentration increases, epalrestat presents significant inhibition of AR, which demonstrates that the system is stable and reliable.

Table 3: Inhibition rate of epalrestat on AR (mean± SD, n = 3)

Concentration (mol/L)	10 ⁻⁸	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵
Inhibition rate (%)	5.61 ± 0.78	24.33 ± 0.55	53.50 ± 0.25	98.80 ± 0.46

Inhibitory Mechanism of PTSS on AR

PTSS3, the one with good inhibitory effect on AR activity, was chosen as inhibitor. Double-reciprocal plot was used to determine the inhibition types on AR at 0.05 and 0.10 mg/mL concentrations. The results shown in Fig. 3 indicate that as the concentration of substrate DL-glyceraldehyde increases, the reaction rate grows. The AR catalysis of the substrate decreases as the polyphenol concentration increases ($r > 0.99$), but the gradient remains unchanged, which suggests that the inhibitory effect of PTSS on AR is uncompetitive inhibition (Li et al., 2014) and that the inhibitors combine with the enzyme-substrate to inhibit enzymatic reactions. The results of Michaelis constant (K_m) and maximum reaction rate (V_{max}) are shown in Table 4. As the inhibitor concentration increases, K_m and V_{max} decrease gradually, manifesting the inhibitory effects on enzymatic reaction.

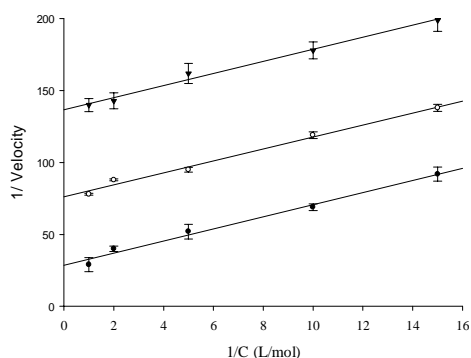


Figure 3: Inhibition type of the polyphenols on AR

Table 4: Kinetic properties of rabbit lens AR

	without inhibitor	PTSS3 (0.05 mg/mL)	PTSS3(0.10 mg/mL)
K_m (mmol/L)	0.1525	0.0543	0.0310
V_{max}	0.0358	0.0131	0.0073

Correlation Analysis of Antioxidant Activity and AR Inhibition

The results indicate that the PTSS antioxidant activity and AR inhibition are significantly correlated, and the correlation coefficients at three concentrations are 0.9924 ± 0.0066 .

Discussion

Polyol pathway is one of the vital ways of glucose metabolism in organisms. To diabetic affected by high-glucose environment *in vivo*, carbohydrate oxidation increases and PP together with other pathological pathways are activated. The occurrences lead to the unbalanced oxidation and anti-oxidization of organisms and the excessive active oxygen radicals, which cause damages to tissues and cells, finally resulting in eye diseases, kidney diseases, neuropathy, and other DM complications. In addition, sustained hyperglycemia causes AR activity to significantly increase, which in turn activates PP and enables a significant amount of the metabolites of sorbitol to accumulate in the cells and affect cellular

structure and function. This phenomenon finally triggers a series of pathological changes in DM. Accordingly; natural antioxidants with AR inhibition activity have the potential for treating DM and DM complications and the advantages of high efficiency and low toxicity. However, studies on the anti-oxidation and AR inhibition activities of natural products are rare (Chung et al., 2005). Therefore, new insights were explored to investigate their correlation and application and to exploit new active materials for preventing and treating DM and DM complications

There are already reports on the antioxidant activity of PTSS (Yang et al., 2014), but its material base remains unknown. However, no report is available about the effects of PTSS on AR inhibition. Three polyphenol extracts were prepared by separating and purifying PTSS to investigate their antioxidant activity and AR inhibition. The PTSS prepared with the experimental method has good antioxidant activity and AR inhibition. PTSS3 eluted by 60 % alcohol exhibited the highest activity, being better than total PTSS. The regression analysis indicates that the AR inhibition and antioxidant activity of PTSS are well correlated, may be due to antioxidant substance interfere the oxidization of coenzyme NADPH and further interfere the AR's catalysis of substrate. The finding suggests that PTSS multiply interferes PP (Giacco & Brownlee, 2010), which possibly affects the occurrence and development of DM and DM complications. However, whether active polyphenols have good anti-oxidant activity and whether natural antioxidant substances have AR inhibition activity require further research.

The research finds that the antioxidant activities of the three polyphenols from *T. sinensis* seeds are not consistent with their polyphenol contents, which shows that the polyphenol constituents differ from the structure (Sun et al., 2014). Hence, polyphenol monomers were separated and purified to analyze the relation between the structure and drug effects for exploring new and high-efficient natural antioxidant substances.

In the experiment on the inhibitory effects of PTSS on AR in the research, rabbit lenses were taken to prepare AR, which is easy, simple, and convenient for sampling. Compared with the large preparation volume of rat lens, it is more applicable for the experiment on AR inhibition *in vitro*. In fact, many synthetic AR inhibitors (ARI) have been developed as drug candidates but virtually most have not been successful in clinical trials due to adverse pharmacokinetic properties, inadequate efficacy, and toxic side-effects. By contrast, PTSS, as a natural antioxidant with AR inhibition activity has potential for treating DN and the advantages of high efficiency and low toxicity. The inhibitory effect of PTSS3 on AR is uncompetitive inhibition, which means that inhibitors combine with enzyme-substrate to inhibit enzymatic reactions. This observation is rare in enzymology but common in research on natural product ARI. The reaction completely differs from the mechanism of epalrestat, which is a reversible non-competitive ARI. This finding means that enzymes can combine with substrates and inhibitors at the same time, leading to the decrease in enzyme activity. The investigation of inhibition mechanisms provides theoretical basis for studying the inhibitory effects of drugs on AR and the development of new inhibitors.

In sum, adjusting the PP mechanisms for PTSS, which was extracted by acetone and purified by macroporous resin, is possible via anti-oxidation and inhibition of AR activity to interfere the occurrence and development of DM complications. The research results also provide theoretical basis for the further separation and preparation of active elements or components from *T. sinensis* seeds. Producers embarking on a much-needed increase in crop cultivation are therefore highly attractive, and the added value and comprehensive utilization of *T. sinensis* is of positive significance.

Acknowledgments

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Conflict of Interest Statement: We declare that we have no conflict of interest.

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