Trop J Nat Prod Res, August 2025; 9(8): 3757 - 3764 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

In vitro Evaluation of Antiglycation, Antioxidation, and α-Glucosidase Inhibitory Activities of Ethanol Extracts of *Terminalia Bellirica* and *Terminalia Chebula* Fruits

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ARTICLE INFO

Article history:
Received 21 May 2025
Revised 20 June 2025
Accepted 26 June 2025
Published online 01 September 2025

ABSTRACT

Accumulation of advanced glycation end-products (AGEs) due to hyperglycemia and oxidative stress contributes to diabetic complications. *Terminalia bellirica* and *Terminalia chebula* are traditional medicinal fruits rich in bioactive phytochemicals with various pharmacological effects. This study assesses their hydroethanolic extracts (TBE and TCE) for phytochemical content, antiglycation, antioxidation, and α-glucosidase inhibitory activities, highlighting their potential for diabetes management. Total phenolics and tannins were measured by the Folin-Ciocalteu method, and flavonoids by aluminum chloride assay. Antiglycation activities were assessed with a bovine serum albumin/fructose model. Superoxide (SO) anion radical inhibition was measured through a riboflavin-light-nitroblue tetrazolium assay, while metal chelation activity was determined using an iron-ferrozine assay. Alpha-glucosidase inhibition was assessed with *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG) substrate. TBE showed significantly higher phenolics (516.44 ± 2.08 mg GAE/g) and tannins (591.0 ± 8.43 mg TAE/g) than TCE, indicating its superior phytochemical content ($p < 0.01$). Regarding antiglycation activity, TBE showed significantly lower IC_{50} for dicarbonyl (2.03 ± 0.21 µg/mL) and AGEs (16.56 ± 4.44 µg/mL) than TCE (4.15 ± 0.20 µg/mL and 37.71 ± 1.14 µg/mL), indicating stronger antiglycation activity ($p < 0.05$). Moreover, TBE demonstrated greater effectiveness in both SO scavenging and ferric ion chelation assays ($p < 0.05$). In the α-glucosidase inhibition assay, both extracts exhibited superior efficacy compared to the standard acarbose ($p < 0.01$). These findings suggest that TBE possesses strong antiglycation, antioxidation, and α-glucosidase inhibitory activities, indicating its potential as a natural agent for managing oxidative stress-related diabetic complications. Further research is warranted to explore its mechanisms and therapeutic applications. (249 words)

Keywords: *Terminalia bellirica*, *Terminalia chebula*, Phytochemicals, antiglycation, antioxidation, α-glucosidase

Introduction

The rising incidence of non-communicable diseases (NCDs) is significantly associated with poor dietary habits, particularly the overconsumption of refined carbohydrates and sugary drinks.[1] NCDs, including type 2 diabetes, cerebrovascular disorders, and cardiovascular illnesses, represent a major cause of global morbidity and mortality.[2] Consequently, there is an urgent need for improved prevention and management strategies. Plant-derived natural products, especially those rich in phytochemicals, present promising opportunities for reducing these risks. This study examines the 95% ethanol extracts of *Terminalia bellirica* (*T. bellirica*) and *Terminalia chebula* (*T. chebula*) fruits, which are traditionally used in Thai Ayurvedic herbal formulas such as Triphala, Trisamor, and Chatuphalathika.

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Citation: Cushnie B, Jaruchotikamol A, Rinthong P, Wichai N, Chantawong T, Phitsaphan K. *In Vitro* Evaluation of Antiglycation, Antioxidation, and α-Glucosidase Inhibitory Activities of Ethanol Extracts Of *Terminalia Bellirica* And *Terminalia Chebula* Fruits. Trop J Nat Prod Res. 2025; 9(8): 3757 – 3764 https://doi.org/10.26538/tjnpr/v9i8.35

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

These fruits are known for their medicinal properties, such as anti-inflammatory, antimicrobial, and antioxidation effects. They have been used traditionally to treat various ailments, including digestive issues, inflammatory conditions, and infections.[3] *T. bellirica* contains various bioactive phytochemicals, including chebulagic acid, chebulanic acid, gallic acid, ellagic acid, and arjunolic acid, which contribute to its antimicrobial, anticancer, and anti-inflammatory properties.[4] Similarly, *T. chebula* is rich in tannins, including gallic acid, ellagic acid, chebulic acid, chebulagic acid, and chebulanic acid, which provide antiallergic, antidiabetic, antioxidation, and immunostimulant benefits.[5] These fruits have been shown to protect against oxidative stress and associated NCDs through free radical scavenging and antioxidant mechanisms.[6]

Dicarbonyls are highly reactive compounds that act as key precursors of advanced glycation end-products (AGEs), which are strongly linked to diabetes-related complications and other NCDs often worsened by poor diets. AGEs form through non-enzymatic Maillard reactions, in which sugars modify proteins and trigger inflammation and oxidative stress.[7,8] Investigating natural antiglycation agents from the fruits of *T. bellirica* and *T. chebula* may therefore offer a proactive approach to diabetes management.

Oxidative stress, driven by reactive oxygen species (ROS), plays a significant role in the development of diabetes and its associated complications. The imbalance between ROS production and the body's antioxidant defenses leads to cellular damage and impaired function, which adversely affects insulin sensitivity, pancreatic beta-cell function, and vascular integrity.[9,10] The hydroethanolic extracts of these fruits may exhibit antiglycation and antioxidation properties, as suggested by their capacity to scavenge free radicals.

Furthermore, their potential to inhibit α-glucosidase, a key enzyme in glucose absorption regulation, is crucial for diabetes management. Understanding these inhibitory effects could offer therapeutic benefits for individuals at risk of type 2 diabetes. The novelty of this research lies in its comprehensive evaluation of *T. bellirica* and *T. chebula* fruits for their potential role in preventing and managing NCDs, focusing on their antiglycation, antioxidation, and α-glucosidase inhibitory activities. While previous studies have highlighted their individual medicinal properties, this work combines these aspects to explore their multifunctional roles in targeting key mechanisms like advanced glycation end-products, oxidative stress, and glucose absorption. By linking traditional uses with modern scientific insights, it offers novel evidence for these fruits as natural agents with therapeutic potential, filling a critical gap in alternative strategies for diabetes and NCD management.

Materials and Methods

Plant Materials and Chemicals

Dried fruits of *T. bellirica* and *T. chebula* were purchased in August 2024 from a licensed herbal medicine shop, Thangsen Siam Yong Co., Ltd., located in Soi Ram Maitri, Pom Prap Sattru Phai District, Bangkok, Thailand. A botanist authenticated these plant materials. Voucher samples are stored at the Faculty of Pharmacy, Mahasarakham University, under voucher numbers PHTB01 and PHTC01.

Gallic acid and aminoguanidine hydrochloride were procured from Thermo Fisher Scientific in Belgium. Aluminum chloride hexahydrate, ascorbic acid, D-fructose, sodium azide, ethylenediaminetetraacetic acid (EDTA), and sodium carbonate anhydrous were sourced from Carlo Erba in Italy. Alpha-glucosidase enzyme type I from *Saccharomyces cerevisiae*, acarbose, nitroblue tetrazolium chloride, and *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG) were obtained from Sigma-Aldrich in the USA. Quercetin hydrate, ferrozine Iron reagent-hydrate, and Girard-T reagent were acquired from Acros Organics in Belgium. Bovine serum albumin (BSA) was purchased from Himedia in Belgium.

The plant materials, with the pulp separated from the seeds, were ground into fine powder. Two hundred grams of *T. bellirica* and *T. chebula* powder were then macerated in 1 L of 95% (v/v) ethanol. Samples were sonicated for 15 min at room temperature using an ultrasonic water bath. The extracts were subsequently maintained at room temperature for 24 h. Filtration was performed using Whatman No.1 filter paper, and the residues were re-extracted three more times with fresh solvent, following the same procedure.[11] The combined filtrates of each sample were evaporated using a rotary evaporator (Rotavapor R-300, Buchi™, Switzerland), and the remaining substances were dried using a freeze dryer (Scanvac CoolSafe 110-4, Labogene™, Denmark). The hydroethanolic extracts of *T. bellirica* and *T. chebula* were designated as TBE and TCE, respectively. The extracts were stored at -20°C until further analysis.

Quantitative Phytochemical Analysis

Evaluation of Total Phenolic Content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method on a 96-well plate.[12] The reaction mixture included 20 µL of extract solution (1 mg/mL), 100 µL of 10% (v/v) Folin-Ciocalteu's reagent dissolved in distilled water, and 80 µL of a 7.5% (w/v) sodium carbonate solution. Gallic acid served as the reference phenolic compound. The mixture was incubated at room temperature in the dark for 30 min. Subsequently, optical density was measured at 765 nm using a microplate reader (Varioskan LUX, Thermo Scientific™, USA). TPC was calculated from a standard curve based on gallic acid, with results expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g).

Evaluation of Total Flavonoid Content

Total flavonoid content (TFC) was assessed using a modified aluminum chloride colorimetric assay.[13] In this method, 500 µL of the extract (1 mg/mL) was mixed with 75 µL of a 5% (w/v) sodium nitrite solution and 150 µL of a 10% (w/v) aluminum chloride solution. The mixture was then incubated in the dark for 5 min. Following this incubation, 500 µL of 1 M NaOH and 275 µL of distilled water were added. Quercetin was used as the standard flavonoid. After an additional 15-min incubation, the optical density of the mixture was measured at 510 nm using a microplate reader (Varioskan LUX, Thermo Scientific™, USA). TFC was calculated based on a standard curve generated from quercetin, with results expressed in milligrams of quercetin equivalents per gram of dry extract (mg QE/g).

Evaluation of Total Tannin Content

Total tannin content (TTC) was evaluated using the Folin-Ciocalteu method.[14] An aliquot of 0.2 mL from each sample (1 mg/mL) was combined with 2.5 mL of distilled water and 0.2 mL of 10% (v/v) Folin-Ciocalteu reagent. This was followed by the addition of 2 mL of a 7% (w/v) sodium carbonate solution. Tannic acid served as the standard tannin. The mixture was incubated at room temperature for 90 min. Subsequently, absorbance was measured at 748 nm using a microplate reader (Varioskan LUX, Thermo Scientific™, USA). TTC was determined using a standard curve established with tannic acid, with results expressed as milligrams of tannic acid equivalents per gram of dry extract (mg TAE/g).

Determination of Antiglycation Activities

Preparation of Glycated Sample

The antiglycation properties of the extract were analyzed using a simulated glycation solution comprising BSA and fructose.[15] Initially, BSA and fructose solutions were prepared in 0.1 M phosphate-buffered saline (PBS) at pH 7.4, which contained 0.02% (w/v) sodium azide to prevent bacterial contamination. Subsequently, a mixture containing 350 µL of 1.1 M fructose solution, 600 µL of BSA solution (50 mg/mL), and 50 µL of the extract at different concentrations (final range 50–1000 µg/mL) was incubated at 37°C for 14 days. This mixture was termed the glycated sample. Aminoguanidine (AG), a synthetic antiglycation agent, was used as the positive control at varying concentrations.

Determination of Dicarbonyl Compounds

The Girard-T reagent was employed to assess dicarbonyl concentration in glycated samples, adhering to a previously described method with minor modifications.[16] Fifty microliters of glycated samples were mixed with 40 µL of 0.5 M Girard-T reagent, which was dissolved in a sodium formate buffer at pH 2.9. The mixture was then incubated at room temperature for 10 min. Subsequently, absorbance was measured at 290 nm using a microplate reader (Varioskan LUX, Thermo Scientific™, USA). The percentage inhibition of dicarbonyl compounds was calculated using equation (1).

$$\text{Percent inhibition} = \frac{[A_{control} - (A_{sample} - A_{blank})]}{A_{control}} \times 100 \quad \text{.....(1)}$$

where $A_{control}$ represents the absorbance of the control reaction and contains all reagents excluding the extract; A_{sample} represents the absorbance of the sample when the extract is present; and A_{blank} represents the absorbance of the sample blank, which consists of absolute ethanol added to the extract without the Girard-T derivative. The blank is used to zero the spectrophotometer and correct for any absorbance due to the solvent or other components of the solution, leaving only the absorbance of the analyte of interest.

Determination of Fluorescent AGEs

Fluorescent AGEs in the glycated samples were quantified by transferring 200 µL of each sample to a black 96-well plate. Fluorescence was measured using a microplate reader (Varioskan LUX, Thermo Scientific™, USA) at excitation and emission wavelengths of 355 nm and 460 nm, respectively. The percentage inhibition of glycation was then calculated using equation (2).

$$\text{Percent inhibition of glycation} = \frac{[(FC - FCB) - (FS - FSB)]}{FC - FCB} \times 100 \quad \text{.....(2)}$$

where FC designates the fluorescence measured in the glycated control absent any sample, and FCB represents the fluorescence of the blank control, which consists of the reaction mixture of BSA absent fructose.

FS indicates the fluorescence of the glycated sample, while FSB signifies the fluorescence of the blank sample, containing the reaction mixture of BSA and a sample absent of fructose.

Determination of Antioxidation Activities
Superoxide Radical Scavenging Assay
A modified riboflavin-light-nitroblue tetrazolium assay was used to assess the scavenging capacity of superoxide (O2•) anion radicals. This method relies on the extract's ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT).[17] The reaction mixture consisted of 20 µL of 750 µM NBT, 20 µL of 1 mM EDTA, 100 µL of 266 µM riboflavin, 20 µL of 50 mM potassium phosphate buffer (pH 7.4), and 40 µL of the sample solution (final concentrations 50–1000 µg/mL), along with reference agents such as ascorbic acid and gallic acid. After a 10-min exposure to fluorescent light, the absorbance was measured at 590 nm using a microplate reader (Varioskan LUX, Thermo Scientific™, USA). The percentage inhibition of superoxide radical production was then calculated using equation (1).

Metal-Chelating Assay
The chelating capacity of ferrous iron was assessed using the protocol of Gulcin *et al.* (2022), with minor modifications.[18] Fifty microliters of the sample (final concentrations 50–2000 µg/mL) or standard EDTA (final concentrations 10–240 µg/mL) were combined with 50 µL of 1 mM $FeSO_4$. The reaction mixture was incubated for 10 min at room temperature. Following this, 100 µL of 0.2 mM ferrozine was added. The mixture was then incubated again for 10 min at room temperature. Absorbance was measured at 562 nm using a microplate reader (Varioskan LUX, Thermo Scientific™, USA). The percentage inhibition of Fe^{2+}-ferrozine complex formation was determined using equation (1).

Determination of α-glucosidase Inhibition
The evaluation of α-glucosidase (*Saccharomyces cerevisiae*) inhibitory activity was performed with slight modifications to a previously established method.[19] In this assay, 50 µL of either the sample or the standard acarbose (final concentrations 0.5–50 µg/mL) was mixed with 100 µL of α-glucosidase enzyme, prepared at a concentration of 0.05 units/mL in phosphate buffer (pH 6.8). The reaction mixture was incubated at 37°C for 10 min. Following this, 50 µL of 0.1 M *p*-NPG substrate was added, and the mixture was incubated for an additional 20 min at 37°C. To stop the reaction, 50 µL of 0.1 M sodium bicarbonate solution was introduced. Absorbance was measured at 405 nm using a microplate reader (Varioskan LUX, Thermo Scientific™, USA). The percentage inhibition of α-glucosidase was calculated using equation (1).

Statistical Analysis
The data are presented as the mean ± standard error of the mean (SEM), while the IC_{50} values are expressed as the mean ± standard deviation (SD), both derived from three independent experiments. Statistical analysis utilized one-way analysis of variance (ANOVA), followed by Tukey's post hoc multiple comparisons test. A significance level of $p < 0.05$ was established to evaluate statistical significance, and the analysis was conducted using GraphPad Prism (version 9).

Results and Discussion

Extraction Yield
This study utilized a combination of maceration and sonication with 95% ethanol as the solvent to extract substances from *T. bellirica* (TBE) and *T. chebula* (TCE) fruits, a method termed ethanolic sonication extraction. This technique resulted in extraction yields of 23.55% and 16.55%, respectively, based on the dry weight of the samples. Compared to traditional maceration, this method enhances extraction yields and enables the recovery of a broader range of compounds from the plant material.[20] These findings are consistent with the research conducted by Singh *et al.* who used the same extraction technique on various parts of the *Terminalia* species to profile phenolic compounds. They reported that *T. chebula* fruit had the highest TPC at 29.41% (w/w), while *T. bellirica* fruit contained 10.18% (w/w).[11]

Phytochemical Analysis
Total Phenolic Content
Figure 1A displays the TPC of the extracts, determined through the Folin-Ciocalteu method. The results are expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g), derived from the standard curve. The findings indicate a significant difference ($p < 0.01$) in TPC between the extracts. TBE has 516.44 ± 2.08 mg GAE/g, whereas TCE contains 264.66 ± 2.39 mg GAE/g. Previous studies in Thailand evaluated TPC in *T. bellirica* and *T. chebula* fruits using 95% ethanol maceration for 3 and 5 days, respectively, without a sonication step before extraction.[21,22] The TPC in TBE was significantly higher than in TCE. Moe *et al.* assessed Myanmar medicinal plants using 95% ethanol maceration for 30 days, also without sonication. Their research showed a TPC of 203 ± 0.01 mg GAE/g in *T. chebula* fruit, aligning with our results. However, the TPC in *T. bellirica* fruit was 2.7 times lower (191.18 ± 0.03 mg GAE/g) than the value observed in our study.[23] Variability in TPC of plant extracts is influenced by factors such as cultivation duration, geographical origin, and extraction methods. Additional considerations include species and maturity stage, which also affect TPC levels.[24]

Total Flavonoid Content
The TFC in the extracts was determined using the aluminum chloride colorimetric assay and reported as milligrams of quercetin equivalents per gram of dry extract (mg QE/g). There was no statistically significant difference in TFC between TBE and TCE (Figure 1B). The observed TFC values were 188.44 ± 5.81 mg QE/g for TBE and 191.42 ± 3.38 mg QE/g for TCE. In line with our findings, Chekdaengphanao *et al.* (2022) also noted no statistical significance, with results showing 265.66 ± 7.31 mg QE/g for TBE and 246.33 ± 3.71 mg QE/g for TCE.[22] Gupta *et al.* analyzed the TPC and TFC of *T. bellirica* fruit extracts and found that the ethyl acetate extract contained higher concentrations of both phenolics and flavonoids compared to aqueous extracts. Specifically, they reported TFC values of 127 ± 0.63 mg QE/g in the aqueous extract and 190 ± 0.97 mg QE/g in the ethyl acetate extract.[25] The TFC in the ethyl acetate extract of *T. bellirica* fruit, as reported by Gupta *et al.*, is similar to the TFC value obtained from the ethanolic extract in our study. Ethyl acetate and ethanol are organic solvents with lower polarity than water and demonstrate a greater ability to dissolve phenolic compounds, particularly those with higher polarity. Among organic solvents, especially in aqueous phases, ethanol is preferred for food and pharmaceutical applications due to legal restrictions and its low toxicity, making it acceptable for human consumption at residual levels compared to other organic solvents.[26]

Total Tannin Content
Figure 1C illustrates the tannin concentrations in extracts, quantified by the Folin-Ciocalteu method and expressed in milligrams of tannic acid equivalents per gram of dry extract (mg TAE/g), measured against a standard curve. The tannin concentration in TBE was significantly higher (591.0 ± 8.43 mg TAE/g) compared to TCE (378.8 ± 9.53 mg TAE/g, $p < 0.01$). These findings corroborate previous HPLC analyses, which confirmed the presence of tannic acid in the hydroethanolic extracts of *T. bellirica* and *T. chebula* fruits. *T. bellirica* contained 278.75 ± 1.41 µg/mL, whereas *T. chebula* contained 116.64 ± 3.14 µg/mL ($p < 0.05$).[22] Tannins, a diverse group of polyphenolic compounds found in plants, are generally classified as hydrolyzable, condensed, or complex. *T. bellirica* and *T. chebula* fruits are particularly abundant in hydrolyzable tannins, such as tannic acid, chebulagic acid, and chebulinic acid. These compounds are known for their extensive pharmacological properties, including antioxidation, anti-inflammatory, antidiabetic, and antimicrobial effects, often due to their abilities to scavenge free radicals, inhibit enzymes, and modulate signaling pathways.[27]

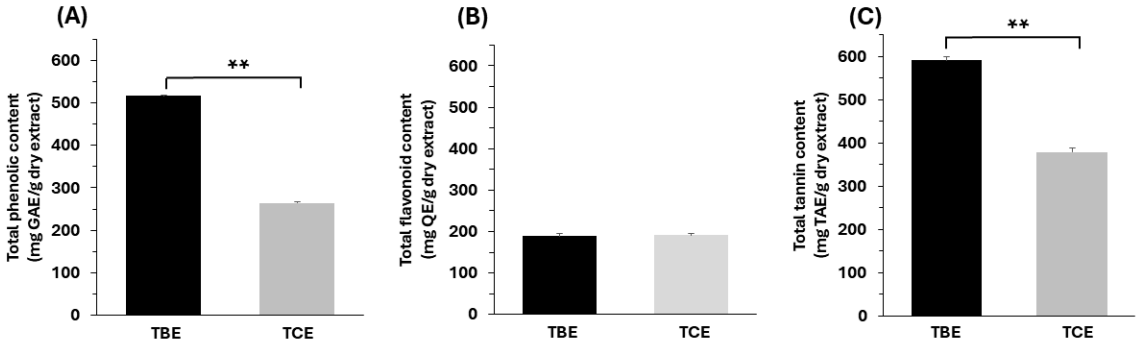

Figure 1: Phytochemical analysis of ethanolic extracts from *T. bellirica* (TBE) and *T. chebula* (TCE) fruits. Measurements include (A) total phenolic content, (B) total flavonoid content, and (C) total tannin content. Data are presented as mean ± SEM (n=3). Statistical significance is denoted by $^{**}p < 0.01$
Abbreviations**:** GAE/g: gallic acid equivalents per gram; QE/g: quercetin equivalents per gram; TAE: tannic acid equivalents per gram

In Vitro Assays for Antiglycation Activities
To investigate the formation of dicarbonyl compounds and fluorescent advanced glycation end-products (AGEs), we employed an *in vitro* BSA/fructose model that closely mimics physiological conditions. We incubated a solution of fructose and BSA at 37°C for 14 days. Aminoguanidine (AG), a well-known antiglycation inhibitor, served as a positive control for comparison with the extracted samples.

Dicarbonyl Inhibition
Figure 2A illustrates the concentration-dependent dicarbonyl inhibitory activity of TBE and TCE, comparing them with the standard antiglycation agent, aminoguanidine (AG). At concentrations of 100 and 250 µg/mL, TCE displayed significantly greater maximal inhibition than both TBE and AG ($p < 0.05$). The IC_{50} values for dicarbonyl inhibition, shown in Figure 2B, further differentiate these extracts. TBE (2.03 ± 0.21 µg/mL), TCE (4.15 ± 0.20 µg/mL), and AG (0.97 ± 0.02 µg/mL) were all significantly different from each other ($p < 0.05$). These findings contrast with those of Atta *et al.* who reported a much weaker inhibition (13–40%) from AG at higher concentrations (1–5 mg/mL) in a BSA/glucose model over an 18-day incubation.[28] The higher dicarbonyl inhibition observed in our study may result from using a fructose-based model instead of glucose. Fructose enhances fluorescence, cross-linking in glycated protein, and protein polymer formation, suggesting it accelerates advanced glycation end-products (AGEs) production. With its open-chain structure and keto group, fructose is significantly more reactive in Maillard reactions, being 8–10 times more reactive than glucose.[29] While data on the specific dicarbonyl inhibitory effects of *T. bellirica* and *T. chebula* fruit extracts are limited, existing evidence shows that phenolic-rich plant extracts effectively reduce dicarbonyl formation. Potential mechanisms include binding to proteins, trapping reactive dicarbonyls, and preventing Amadori product formation.[30] Future research should aim to elucidate the specific mechanisms of various extracts and evaluate their therapeutic potential for preventing or mitigating glycation-related diseases.

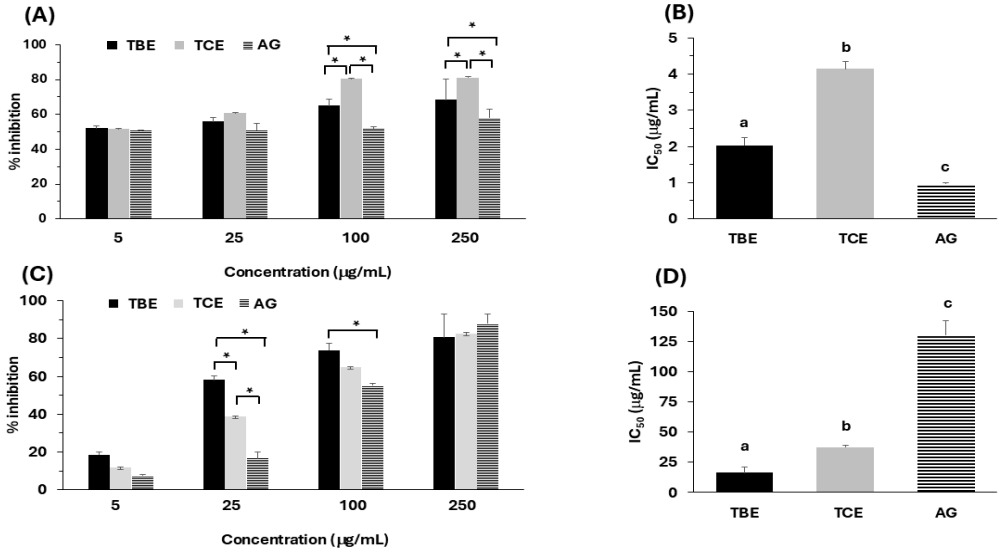

Figure 2: Evaluation of the antiglycation effects of ethanolic extracts from *T. bellirica* (TBE) and *T. chebula* (TCE) fruits. (A) Percentage inhibition of dicarbonyl formation (mean ± SEM). (B) IC_{50} values for dicarbonyl inhibition (mean ± SD). (C) Percentage inhibition of advanced glycation end products (AGEs) formation (mean ± SEM). (D) IC_{50} values for AGEs inhibition (mean ± SD). Statistically significant differences are indicated by $^{*}p < 0.05$. Bars labeled with different letters (a-c) denote significant differences (p-value < 0.05).
Abbreviation**:** AG: aminoguanidine

AGEs Inhibition

Figure 2C illustrates the concentration-dependent inhibition of AGE formation by TBE, TCE, and the standard AG. All three samples exhibited inhibition of AGE formation. At a concentration of 250 µg/mL, their inhibitory effects were similar, with no statistically significant differences observed. However, analysis of IC_{50} values (Figure 2D) revealed notable differences in potency. TBE demonstrated significantly stronger AGE inhibition (IC_{50} 16.56 ± 4.44 µg/mL) than both TCE (IC_{50} 37.71 ± 1.14 µg/mL; $p < 0.05$) and AG (IC_{50} 130.95 ± 11.24 µg/mL; $p < 0.01$). These results corroborate an earlier study, which reported a comparable potency for AG, presenting an IC_{50} value of 124.66 ± 1.99 µg/mL.[31] In a related study by Moe *et al.*, a BSA/fructose glycation assay with a 7-day incubation period was conducted. They found that 95% ethanolic extracts of *T. bellirica* and *T. chebula* fruits, each tested at a final concentration of 50 µg/mL, inhibited AGE formation by 78.96 ± 2.85% and 70.74 ± 2.57%, respectively. For comparison, a 1 mM rutin standard inhibited AGE formation by 83.24 ± 1.73% in their investigation.[23]

The current study shows that TBE more effectively inhibits AGE formation than TCE. This correlates with phytochemical analysis revealing higher levels of phenolics and tannins in TBE. Phenolic compounds are renowned for their antioxidation activity, primarily through their hydroxyl groups, which neutralize reactive species involved in glycation. Because of their high molecular weight and protein-binding ability, tannins enhance antiglycation effects by preventing protein cross-linking. These elevated bioactive compounds make TBE a promising natural agent for preventing or reducing AGE-related health problems, including diabetic complications. Further investigation into specific phenolic compounds (e.g., gallic acid) and tannin constituents (e.g., chebulagic acid, chebulinic acid) could provide deeper insights into their roles and therapeutic potential.[7,28]

In Vitro Assays for Antioxidation Activities

This study evaluated the antioxidation activities of TBE and TCE using superoxide (SO) radical scavenging and metal-chelating assays. These assessments are crucial for determining the extracts' ability to reduce oxidative stress, which is a key factor in developing AGE-related NCDs such as diabetes, cardiovascular diseases, and other metabolic disorders.[32,33]

Superoxide Radical Scavenging Activity

The assessment of superoxide (SO) radical inhibition, determined by the reduction of NBT-formazan, demonstrated the antioxidant activity of both the TBE and TCE extracts, as well as the standard agents, ascorbic acid and gallic acid. Both extracts achieved their peak SO inhibition at a concentration of 500 µg/mL, with TBE and TCE showing inhibition percentages of 86.35 ± 1.04% and 98.47 ± 0.78%, respectively (Figure 3A). The difference in inhibition between these extracts was not statistically significant.

Figure 3B displays the IC_{50} values, indicating the concentration required to scavenge 50% of the SO radicals. TBE had an IC_{50} of 34.66 ± 4.05 µg/mL, while TCE's IC_{50} was 47.64 ± 3.69 µg/mL. Among the standard agents, gallic acid was notably more effective, with an IC_{50} of 6.44 ± 2.29 µg/mL, significantly outperforming ascorbic acid (IC_{50}: 134 ± 4.24 µg/mL; $p < 0.01$).

These findings are consistent with those of Moe *et al.* who examined Myanmar medicinal plants, reporting SO radical inhibition of 86.38 ± 8.05% and 88.56 ± 1.87% at 500 µg/mL for ethanolic extracts of *T. bellirica* and *T. chebula* fruits, respectively, although IC_{50} values were not determined in their study.[23] Furthermore, Chatterjee *et al.* (2024) documented similar SO radical inhibition in 50% methanol extracts of *T. bellirica* and *T. chebula* fruits from West Bengal, India, with IC_{50} values of 13.70 ± 0.81 and 15.74 ± 0.43 µg/mL, respectively.[34] However, their study lacked statistical analysis to substantiate these results.

Metal-chelating Activity

Metal ions, particularly iron (Fe^{3+}), play a role in regulating glucose metabolism and can influence the development of NCDs through the glycation process. Metal-chelating activity, which involves the binding of metal ions to form stable complexes, is crucial for inhibiting metal-

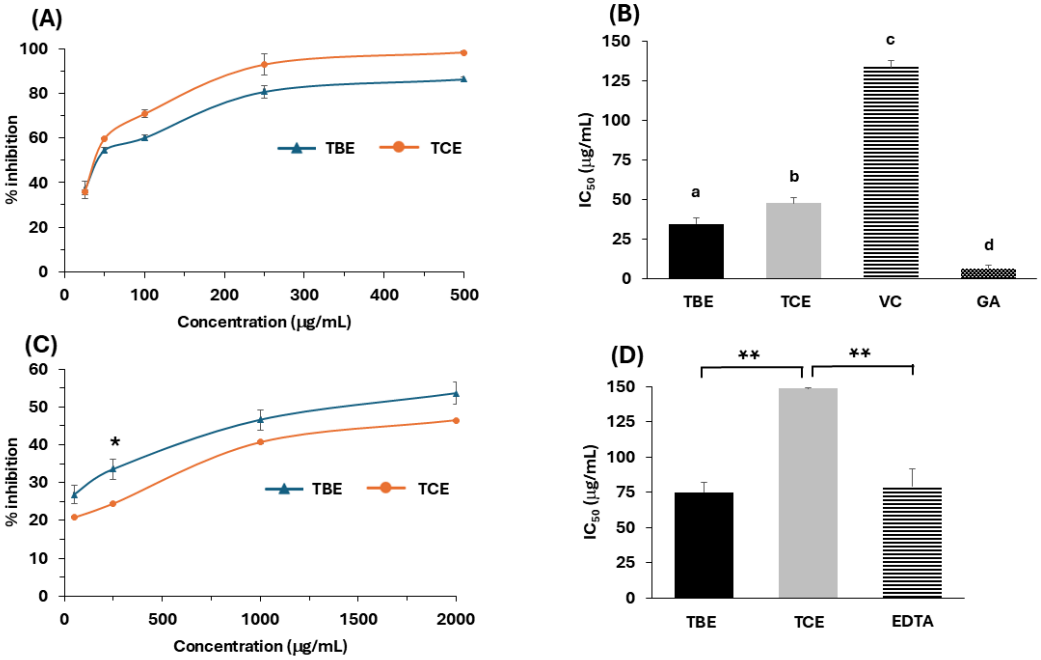

Figure 3: Assessment of the antioxidant properties of ethanolic extracts from *T. bellirica* (TBE) and *T. chebula* (TCE) fruits. (A) Percentage inhibition of superoxide (SO) anion radical scavenging activity (mean ± SEM). (B) IC_{50} values for SO radical scavenging activity (mean ± SD). (C) Percentage inhibition of metal chelating activity (mean ± SEM). (D) IC_{50} values for metal chelating activity (mean ± SD). Significant differences are indicated by $*p < 0.05$ and $**p < 0.01$. Bars with different letters (a-d) represent statistically significant variations ($p < 0.05$). Abbreviations: VC–Vitamin C, GA–Gallic Acid, EDTA–Ethylenediaminetetraacetic acid

catalyzed oxidation reactions. By preventing these reactions, chelating agents impede glycation, a non-enzymatic process where sugars attach to proteins, leading to the formation of advanced glycation end-products (AGEs) associated with various health conditions.[35] Both TBE and TCE displayed their highest metal-chelating effects at 2 mg/mL, with no significant difference in their effectiveness, achieving 53.61% ± 2.9 and 46.46% ± 0.27, respectively (Figure 3C). Figure 3D shows their IC_{50} values, where TBE (75.11 ± 6.79 µg/mL) demonstrated metal-chelating activity comparable to EDTA (79.44 ± 12.07 µg/mL). In contrast, TCE was significantly less effective, with an IC_{50} of 148.6 ± 0.56 µg/mL ($p < 0.01$), indicating lower chelating potency.

A previous study that examined the metal-chelating activity of ethanolic *T. chebula* fruit extract from Pakistan reported an IC_{50} value of 445 ± 0.8 µg/mL, approximately three times higher than the IC_{50} value observed in our current study. Conversely, in that same study, the IC_{50} value for EDTA was 6.50 ± 0.07 µg/mL, which is 12 times more potent than what we found in our study.[36] These findings underscore significant differences in chelating efficacy that may arise from variations in extract preparation, geographic origin, or analytical methods.

A notable methodological difference between the studies is the reagent used for ferrous detection. Khan *et al.* employed ferene, whereas the current study used ferrozine. Structurally, these two reagents differ: ferene contains oxygen atoms in each of its furyl rings, while ferrozine features two phenyl sulfonate groups. The sulfonyl furyl groups in ferene replace these phenyl sulfonate groups found in ferrozine. Additionally, the accuracy of ferrous detection can be influenced by various factors, including pH levels, reagent concentrations, temperature, the presence of competing anions, and the presence of Fe^{3+} ions, all of which can affect the assay's reliability.[37]

In Vitro α-glucosidase Inhibitory Activity

Alpha-glucosidase is a critical enzyme in the small intestine responsible for carbohydrate digestion. It breaks down complex sugars into glucose for absorption. Inhibitors of α-glucosidase, such as acarbose, miglitol, and voglibose, are commonly used to reduce glucose absorption, thus assisting in the management of hyperglycemia in diabetic patients. However, these medications often cause side effects, including abdominal cramping, bloating, and diarrhea. Chronic hyperglycemia leads to increased protein glycation, resulting in glycation stress. This stress activates oxidative and inflammatory signaling pathways, ultimately contributing to various diabetic complications. Thus, natural compounds that can simultaneously inhibit glucose absorption and impede the glycation process hold significant promise for effectively reducing secondary health issues associated with diabetes.[38]

Figure 4A shows the percentage of α-glucosidase inhibition exhibited by the extracts. Both TBE and TCE achieved their maximal inhibitory effects at 25 µg/mL, with values of 98.2 ± 2.02% and 98.58 ± 0.43%, respectively, showing no statistically significant difference between them. Figure 4B displays the IC_{50} values for the extracts and the reference compound acarbose. TBE exhibited a significantly lower IC_{50} value of 3.27 ± 0.12 µg/mL ($p < 0.01$), indicating higher potency, compared to TCE's IC_{50} of 16.55 ± 2.72 µg/mL and acarbose's IC_{50} of 88.39 ± 0.57 µg/mL. This suggests that TBE is a more effective α-glucosidase inhibitor among the samples.

The IC_{50} values observed in this study are higher than those reported by Sabuhom *et al.* for hydroethanolic extracts of *T. bellirica* and *T. chebula*, which were 0.05 ± 0.00 and 0.12 ± 0.06 µg/mL, respectively. In comparison, acarbose exhibited an IC_{50} of 177.78 ± 4.44 µg/mL in their research.[21] Though our findings confirm that TBE is more potent than TCE, both extracts showed higher IC_{50} values than those previously recorded. Notably, the IC_{50} for acarbose in our study was approximately half of that reported by Sabuhom *et al.* This discrepancy may stem from differences in the enzymatic assay conditions, particularly the concentration of α-glucosidase (*Saccharomyces cerevisiae*). Sabuhom *et al.* used an enzyme concentration three times higher than ours, likely affecting the measurements of inhibitory potency. Furthermore, the α-glucosidase inhibitory activity of TBE seems to correlate with its total phenolics and tannins content, suggesting that these phytochemicals contribute to its effectiveness as an inhibitor.

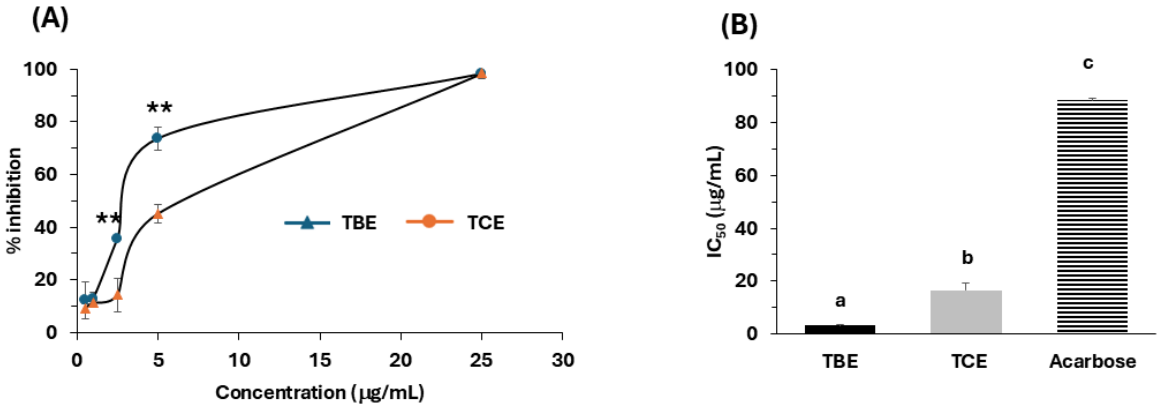

Figure 4: Evaluation of α-glucosidase inhibitory activity of ethanolic extracts from *T. bellirica* (TBE), *T. chebula* (TCE) fruits. (A) Percentage inhibition of α-glucosidase enzyme activity (mean ± SEM). (B) IC_{50} values reflecting the potency of inhibition. Significant differences are demoted by $^{**}p < 0.01$. Bars labeled with different letters (a-c) indicate statistically significant differences ($p < 0.05$).

A prior UPLC-ESI-MS/MS study identified 37 phenolic compounds in various *Terminalia* species, including *T. bellirica* and *T. chebula* fruits from South India. The study used 100% ethanol extraction combined with sonication, similar to the method employed in our current work. It found that the concentrations of chebulagic acid and gallic acid were nearly double in *T. bellirica* fruit compared to *T. chebula* fruit.[11] This finding suggests that *T. bellirica* may have a richer profile of these bioactive phenolics, potentially contributing to its enhanced biological activities.

Chebulagic acid, a prominent hydrolyzable tannin found in *Terminalia* species, is recognized for its antihyperglycemic effects and its efficient inhibition of α-glucosidase.[39] In a similar manner, gallic acid inhibits α-glucosidase by competitively binding to the enzyme's active site, preventing substrate attachment. However, its potency is lower, with an IC_{50} of 250 µg/mL, compared to acarbose, which is more effective with an IC_{50} of 160 µg/mL.[40] These findings underscore the potential of tannins and phenolic acids from *Terminalia* species as natural inhibitors, though their activity varies when compared to standard pharmaceutical agents.

Conclusion

This study provides preliminary *in vitro* evidence that hydroethanolic extracts of *Terminalia bellirica* (TBE) and *Terminalia chebula* (TCE)

fruits exhibit significant antiglycation, antioxidant, and antidiabetic properties. Notably, TBE demonstrates superior efficacy compared to TCE in key areas, such as inhibiting dicarbonyl compound formation, suppressing advanced glycation end-products (AGEs), chelating ferrous ions, scavenging superoxide radicals, and inhibiting α-glucosidase activity. These enhanced activities are likely due to the higher levels of phenolic and tannin compounds in TBE. By preventing free radical-mediated damage, protein oxidation, and glycation, TBE may play a crucial role in shielding proteins from oxidative stress. Overall, TBE shows promise as a natural agent in inhibiting AGE-related pathways, which is particularly beneficial for diabetic patients at increased risk of chronic complications and NCDs. Further research is strongly recommended to focus on TBE's ability to trap methylglyoxal and elucidate the mechanisms underlying its bioactivity to optimize its therapeutic potential.

Conflict of Interest

The authors declare no conflicts of interest.

Authors's Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

This research project was financially supported by the Faculty of Pharmacy at Mahasarakham University, Thailand (S#1/2567). The authors express gratitude to the Pharmaceutical Chemistry and Natural Product Research Unit, Faculty of Pharmacy, Mahasarakham University, Thailand, for providing laboratory equipment and financial assistance.

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