

Extraction, purification, and evaluation of bioactivities of total triterpenoids from Persimmon (*Diospyros kaki* L.f.) Leaves

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ABSTRACT

The current research focused on enhancing the extraction and purification processes of triterpenoids found in persimmon leaves, as well as assessing their biological effects. The extraction yield of triterpenoids was 8.26%. Activated carbon was used to purify the extracts, resulting in an increase in the content of triterpenoids in the final product from 24.43% to 79.66%. The recovery yield of triterpenoids from the ethanol extract was 73.57%. Ethanol was recovered and reused as a solvent. The total triterpenoids exhibited cytotoxic activities against several cancer cell lines, with IC_{50} values of approximately 32.49–55.02 $\mu\text{g/mL}$, as well as anti-inflammatory activities ($IC_{50} = 29.55 \pm 1.67 \mu\text{g/mL}$). The total extract and total triterpenoids demonstrated significant inhibition against α -glucosidase, having IC_{50} values of $0.18 \pm 0.05 \mu\text{g/mL}$ and $2.12 \pm 0.15 \mu\text{g/mL}$, respectively. Additionally, for antioxidant activity, the IC_{50} values of the total extract and total triterpenoids were 12.53 ± 0.20 and $13.92 \pm 0.18 \mu\text{g/mL}$, respectively. The triterpenoids showed higher inhibition of acetylcholinesterase compared with the total extract (IC_{50} : 40.07 ± 2.54 and $112.22 \pm 9.68 \mu\text{g/mL}$, respectively). However, they did not show inhibitory activity against bacteria or fungi. In conclusion, information about the extraction, purification and biological activities of total triterpenoids from persimmon leaves was fully provided for the first time.

1. Introduction

Plant diversity leads to a wide range of secondary metabolites, which play a vital role in the process of discovering and developing drugs [1–4]. According to David et al. [5], from 1981 to 2019, drugs of natural origin accounted for 64.9% of newly approved drugs. Natural compounds have very complex chemical structures and it is too difficult and expensive to synthesise them by chemical methods on an industrial scale. Furthermore, natural products exhibit special features compared to conventional synthetic molecules. Therefore, extracting these bioactive compounds from nature is the most feasible option [6].

The extraction method is one of the principal factors that affect the preservation of the physicochemical and biological properties of the target molecules [7]. The selection of the solvent system and extraction technique relies on the characteristics of the substance intended for

isolation. The extraction process should be high-performance, simple, fast, environmentally safe and economically efficient. Consequently, the extraction of active ingredients from medicinal plants needs appropriate methods to improve extraction yields and increase the content of the target compounds in the extract and the fractions [8,9].

Triterpenoids are organic compounds that contain 30 carbon atoms and are formed by the polymerisation of six isoprene units. Triterpenoids are biosynthesised from squalene and are widely distributed in nature [10]. Based on their chemical structures, triterpenoids are classified into linear, monocyclic, dicyclic, tricyclic, tetracyclic and pentacyclic groups [11]. More than 20,000 triterpenoids structures have been found in plants, animals, fungi, ferns and marine organisms. Triterpenoids are present in either the free state or in combination with different types of sugar-forming glycosides or esters [12]. Triterpenoids exhibit many potential pharmacological effects. They have been described as

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antitumoral, anti-inflammatory, antioxidant, antimicrobial, antiviral, anti-diabetic, acetylcholinesterase inhibitory, immunomodulatory and analgesic agents [12–16]. Currently, triterpenoids are widely used to prepare semisynthetic derivatives as clinically useful anticancer agents [17]. Several natural and semisynthetic derivatives of bioactive triterpenoids are used in pharmaceuticals, cosmetics and foods [13].

Persimmon (*Diospyros kaki* L.f.), originating from China, is a tree categorized within the Ebenaceae family and extensively found across East Asian nations such as Korea, Japan, and Vietnam. Persimmon leaves (PL) have a long history of traditional medicinal use for various purposes, including addressing coughs, chronic ulcers, strokes, constipation, burns, paralysis, hemorrhages and frostbite [18]. In Japan, kakinoha-cha (persimmon leaf tea) has been widely used as a health-promoting beverage and has been considered an anti-aging tea because of its rich vitamin C content [18,19]. The main compounds found in PL are flavonoids, tannins, ionones, naphthoquinones and various triterpenoids [20]. Phuong et al. [21] reported the presence of 14 ursane and oleanane triterpenoids in PL. Jaeyoung et al. [20] isolated 27 compounds, including seven triterpenoids, from PL. We isolated and determined the structures of five triterpenoids (oleanolic acid, ursolic acid, diospyric acid B, rotungenic acid and pomolic acid) from PL in our previous study [22]. Recent studies have demonstrated that PL extracts display various pharmacological activities, such as effects on the cardiovascular system, and antioxidant, anti-atherosclerosis, anti-anaphylactic, antibacterial, anticancer, antitumor, neuroprotective and anti-allergy activities [18,20].

In this study, for the first time, total triterpenoids from PL were extracted, purified and evaluated for biological activities. The response surface methodology (RSM) was employed to optimize the key factors influencing extraction efficiency, including ethanol concentration, liquid-solid ratio, extraction time, and extraction temperature. The total triterpenoids from the extract were purified using activated carbon. The biological activities (cytotoxic activity, antioxidant activity, acetylcholinesterase (AChE) inhibition, antimicrobial, anti-inflammatory and α -glucosidase inhibitory activities) of the total triterpenoids were also assessed.

2. Materials and methods

2.1. Chemicals and materials

Persimmon leaves were collected in Thua Thien Hue Province, Vietnam, in September 2022 and were subsequently identified by experts from the Vietnam National Museum of Nature.

Ethanol (99.5%, AR) was purchased from Chemical And Scientific Technological Materials Co., Ltd. (Ho Chi Minh, Vietnam). Acetic acid ($\geq 99.5\%$, AR), and sulfuric acid (95–98%, AR) were purchased from Xilong Scientific Co., Ltd. (Guangdong, China). Ursolic acid, Tris base, dimethyl sulphoxide (DMSO), enzyme α -glucosidase, p-Nitrophenyl α -D-glucopyranoside (pNPG), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), quercetin, Lipopolysaccharide (LPS), sulfanilamide, Thiazolyl blue tetrazolium bromide (MTT), sodium nitrite, phosphoric acid, N-1-naphthylethylenediamine, N^G-Methyl-L-arginine acetate (L-NMMA), acetylcholinesterase, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCI), ampicillin, cefotaxime, nystatin, and galantamine were supplied by Sigma-Aldrich (Missouri, USA). Dulbecco's modified Eagle's medium (DMEM), sodium bicarbonate (NaHCO₃), L-glutamine, fetal bovine serum (FBS), Trypsin-EDTA, trichloroacetic acid (TCA) were sourced from Thermo Fisher Scientific (Massachusetts, USA). Ellipticine was procured AK Scientific, Co. (California, USA). RAW 264.7 cell lines were supplied by the University of Perugia, Italy. Cancer cell lines including human hepatocellular carcinoma (HepG2), human breast carcinoma (MCF-7), human colon carcinoma (HT-29), human colonic carcinoma (SW480), lung carcinoma (SK-LU-1), human cervical carcinoma (Hela), human gastric carcinoma (AGS), and human embryonic kidney (HEK-293A) normal cells were obtained from

University of Milan, Italy and Long Island University, USA. The microorganisms, namely *Staphylococcus aureus* ATCC 13709 (*S. aureus*), *Pseudomonas aeruginosa* ATCC 15442 (*P. aeruginosa*), *Bacillus subtilis* ATCC 6633 (*B. subtilis*), *Escherichia coli* ATCC 25922 (*E. coli*), and *Candida albicans* (C. albicans) ATCC 10231, were obtained from the American Type Culture Collection. *Lactobacillus fermentum* N4 (*L. fermentum*) and *Salmonella enterica* (*S. enterica*) were acquired from the Vietnam Type Culture Collection.

2.2. Total triterpenoids content assay

The method previously outlined by Wei et al. [23] was employed to quantify the total triterpenoids content in PL. Initially, a 0.2 mL sample solution was dried using a water bath at 70 °C. Subsequently, 0.2 mL of a freshly prepared solution containing 5% vanillin in glacial acetic acid (w/v) and 1.8 mL of sulfuric acid were added. The solution obtained was subjected to incubation in a water bath at 70 °C for a duration of 30 min. Afterward, the mixture was rapidly cooled and then brought to a final volume of 10 mL using glacial acetic acid. The UV/VIS spectrophotometer was utilized to measure the absorbance at 573 nm. Total triterpenoids yield was calculated according to the equation below, and outcomes were presented as grams of ursolic acid equivalent per 100 g of dry weight (% g/g).

$$\text{Total triterpenoids yield} = \frac{C \cdot n \cdot V}{m \cdot 1000} \cdot 100\%$$

Here, C represents the concentration of triterpenoids calculated based on the ursolic acid standard curve ($\mu\text{g/mL}$); n is the dilution factor; V is the volume of the obtained extract (mL); and m is the weight of medicinal materials (mg).

2.3. HPLC

The purity of the total triterpenoids extract was confirmed by a reversed-phase HPLC system (Shimadzu, Kyoto, Japan) with a PDA detector, using the standards of ursolic acid, oleanolic acid (Sigma-Aldrich), diospyric acid B, rotungenic acid and pomolic acid (isolated compounds from PL were used as standards) [22]. Triterpenoids were separated using a Zorbax Eclipse XDB C18 reversed-phase chromatographic column (4.6 \times 150 mm i.d., 5 μm) with a mobile phase consisting of methanol: 0.1% H₃PO₄ aqueous solution (88:12, v/v). The flow rate and the maximum absorption wavelength were 1 mL/min and 210 nm, respectively. The relative purity of the total triterpenoids extract was determined as a percentage of the peak areas of the five standard triterpenoids in relation to the total peak areas obtained at 210 nm.

2.4. Extraction process

2.0 g of PL powder was measured and subjected to reflux extraction in an ethanol-water solution under varying influencing factors. These factors included different ethanol concentrations (20%, 40%, 60%, 80%, and 96%), liquid-solid ratios (1:10, 1:15, 1:20, 1:25, and 1:30 g/mL), extraction temperatures (50 °C, 60 °C, 70 °C, 80 °C, 90 °C, and 100 °C), and extraction times (30, 40, 50, 60, 90, and 120 min). The resulting extraction mixtures were subsequently filtered using a Buchner funnel for further analysis. Each experiment was conducted in triplicate, and the outcomes were presented as the mean \pm standard deviation.

2.5. Response surface methodology experimental design

The Box-Behnken design (BBD) was used to optimize the extraction parameters for total triterpenoids. The independent variables consisted of ethanol concentration (A), liquid-solid ratio (B), extraction time (C), and extraction temperature (D). The efficiency of total triterpenoids extraction was selected as the dependent variable. The parameter levels were presented in Table S1. In the experimental design, a total of 29

experiments were conducted, consisting of five replicates of the central point and 24 factorial points. Each point within the experimental design was replicated three times, and the resulting responses were presented as mean values. Design-Expert (version 13.0, Stat-Ease Inc., Minnesota, USA) was utilized to identify the optimal conditions. A p-value below 0.05 was regarded as statistically significant.

2.6. Purification of triterpenoids and recycling of the solvent

The collected extract (300 mL) was subjected to evaporation using a vacuum evaporator to yield a liquid suspension (1:1 g/mL). One part of the liquid syrup was mixed with three parts of distilled water, leading to the formation of a precipitate within 24 h. The resulting precipitate was obtained through filtration using a Buchner funnel, which effectively eliminated water-soluble impurities such as gum, mucus, pectin, and polysaccharides. The purification process of total triterpenoids involved the use of activated carbon. Ethanol was utilized to dissolve the precipitate, and varying amounts of activated carbon (1, 2, 4, 6, 8, and 10 g) were employed to adsorb the pigments. Following filtration through a Buchner funnel, a clear liquid was obtained. The solvent was then recovered, resulting in a white, powdery mixture of triterpenoids. To assess the method's efficacy, the percentages of triterpenoids before and after impurity removal, as well as the purity of the obtained triterpenoids, were determined. For solvent recycling, all ethanol employed in the extraction and purification processes was reclaimed for future solvent utilization. The concentration of the reclaimed ethanol was gauged using alcohol meters (Amarell, Germany). Subsequently, the reclaimed alcohol was blended with distilled water to attain a solution containing 63% alcohol. The suitability of the reclaimed alcohol for reuse was assessed through three consecutive extraction cycles involving fresh biomass. Each experiment was replicated three times.

2.7. Cytotoxicity activity

The cytotoxic effects on the growth of human cancer and normal cell lines, including AGS, MCF-7, SK-LU-1, HT-29, HepG2, SW480, HeLa, and HEK-293A, were assessed using the SRB method. These eight cell lines were cultured in 50 mL of DMEM supplemented with 2 mM L-glutamine, 1.5 g/L NaHCO₃, and 10% FBS. The growth medium was renewed every 48 h, and cell detachment was carried out using 0.05% trypsin-EDTA. The collected cells were then grown at intervals of 3 to 5 days in a 37 °C incubator with a 5% CO₂ environment. Cell viability was determined via the SRB assay, which depended on the cellular protein content to ascertain cell density. Cells were initially seeded overnight in 96-well microplates at a density of 5700 cells per well. Subsequently, test samples were introduced into each well containing cells. The cells were kept under stable conditions for another three days. After discarding the medium, cell monolayers were fixed using 20% cold TCA at 4 °C for one hour, followed by staining using 1X SRB staining solution for 30 min at room temperature. Following several washes with a 1% acetic acid solution, the dye attached to proteins was solubilized using 10 mM Tris base, and the optical density was determined at 515 nm. Ellipticine was utilized as the positive control in this assay. Inhibition rates (IR) were calculated using the following equation:

$$I\% = [1 - (OD_t - OD_0) / (OD_c - OD_0)] \times 100$$

in which, OD_t corresponds to the mean optical density recorded on the third day, OD₀ represents the mean initial optical density, and OD_c stands for the mean optical density of the blank sample.

2.8. α-Glucosidase inhibiting effect

The α-glucosidase inhibitory effect was evaluated following the method previously outlined [24] with minor adjustments. Briefly, test samples were prepared at various concentrations. Subsequently, 50 μL

of each test sample was incubated with a 100 μL solution of α-glucosidase (0.2 U/mL) in 100 mM phosphate buffer (pH 6.8) for 10 min at 37 °C. Afterward, 50 μL of pNPG was introduced, and the reaction was permitted to proceed for 30 min at a temperature of 37 °C. The reaction was halted by adding 0.1 M Na₂CO₃. The resulting p-nitrophenol release was quantified by measuring absorbance at 410 nm. Acarbose served as the positive control. The percentage of inhibition was determined using the subsequent formula:

$$I\% = (1 - A_{\text{sample}}/A_{\text{control}}) \times 100$$

where A_{sample} and A_{control} represent the enzyme activities in the reaction mixture with and without the test sample, respectively.

2.9. Antioxidant activity

The antioxidant effects were assessed through the DPPH radical scavenging assay, following a method outlined previously with minor adjustments [25]. In the absence of light, a mixture consisting of 2 mL of 0.135 μM DPPH solution and 2 mL of the test sample was kept at 37 °C for half an hour. Subsequently, the absorbance was recorded at 517 nm using a UV-1800 spectrophotometer from Shimadzu, Oregon, USA. The positive control used in the study was quercetin. The inhibitory activity was determined using the formula:

$$I\% = (1 - A_{\text{sample}}/A_{\text{blank}}) \times 100$$

Here, A_{sample} represents the absorbance of the tested mixture, while A_{blank} corresponds to the DPPH absorbance.

2.10. Anti-inflammatory activity

The RAW 264.7 cell line was seeded into 96-well culture plates at a concentration of 2 × 10⁵ cells/well and was kept overnight at 37 °C with 5% CO₂. Subsequently, the media were replaced with FBS-free DMEM and maintained for 3 h. Different concentrations of the test samples were introduced. Following a 2-hour treatment period, the cells were activated with 1 μg/mL of LPS for a duration of 24 h. Nitrite presence was detected using the commercial NO detection kit Griess Reagent System (Promega Corporation, WI, USA) following the manufacturer's instructions. A sodium nitrite (NaNO₂) standard was utilized to establish the calibration curve. L-NMMA served as a positive control. A MTT-based colorimetric assay was performed to assess cell viability in the experiments [26].

2.11. AChE inhibition effect

The AChE inhibitory assay was conducted following Ellman's method [27], with minor modifications. A 10% DMSO solution was employed to dissolve test samples at various concentrations. A mixture comprising phosphate buffer (pH = 8), AChE solution (0.25 U/mL), and the test sample was incubated at room temperature for 15 min. Subsequently, ATCI solution (2.4 mM) and DTNB solution (2.4 mM) were introduced, leading to the formation of a yellow product, 5-thio-2-nitrobenzoate. The absorbance was measured at 405 nm after a 15-minute incubation at room temperature. Galanthamine served as the positive control. The AChE inhibition was represented as IC₅₀ (μg/mL). The inhibition percentage was determined using the formula:

$$I\% = (1 - A_{\text{sample}}/A_{\text{control}}) \times 100$$

where A_{sample} and A_{control} are the enzyme activities in the reaction mixture with and without the test sample.

2.12. Antimicrobial activity

In the present study, a total of 7 strains were utilized, comprising 3 Gram-positive strains (*B. subtilis*, *S. aureus*, and *L. fermentum*), 3 Gram-

negative strains (*E. coli*, *S. enterica*, and *P. aeruginosa*), and 1 *Candida* species strain (*C. albicans*). The sample solutions were prepared by diluting them in DMSO and distilled water at various concentrations. The broth dilution method outlined by Franz et al. was adopted [28]. Reference compounds such as ampicillin, cefotaxime, and nystatin were employed. The IC₅₀ value was ascertained based on the inhibition percentage of microorganism growth. The inhibition percentage was calculated using the following equation:

$$I\% = \frac{(\text{OD}_{\text{positive control}} - \text{OD}_{\text{sample}})}{(\text{OD}_{\text{positive control}} - \text{OD}_{\text{negative control}})} \times 100\%$$

where OD_{positive control}, OD_{negative control}, and OD_{sample} are optical density values of the positive control, negative control, and tested samples, respectively.

2.13. Data analysis

The analysis of variance (ANOVA) was conducted through the utilization of SPSS software. Mean differentiation was assessed employing the least significant difference (LSD) test, considering a significance level of $p < 0.05$. Design-Expert (version 13.0, Stat-Ease Inc., Minnesota, USA) was employed to establish the optimal extraction conditions. The IC₅₀ values were determined using linear regression analysis.

3. Results and discussion

3.1. Optimisation of triterpenoids extraction process from PL

3.1.1. Effect of ethanol concentration on the extraction yield

To select the optimal concentration of ethanol, the triterpenoids extraction efficiency was investigated with different ethanol concentrations (20%, 40%, 60%, 80% and 96%) (Fig. 1A). The results showed that the ethanol concentration affected the extraction yield of triterpenoids from PL. When the content of ethanol increased from 20% to 60%, the extraction yields increased. Subsequently, the extraction efficiency gradually decreased with the increase of alcohol concentration from 60% to 96%. The highest yield of total triterpenoids from PL (6.93 ± 0.20%) was obtained when the ethanol concentration in the

extraction solvent reached 60%. The choice of ethanol concentration affects not only the extraction yields of triterpenoids but also the properties of the obtained triterpenoids. In general, polar triterpenoids are efficiently extracted at lower ethanol concentrations, whereas non-polar triterpenoids are efficiently extracted at higher ethanol concentrations [23]. At low ethanol concentrations, water-soluble ingredients (e.g., polysaccharides and pectins) compete with triterpenoids dissolution, resulting in decreased extraction yields. By contrast, at high ethanol concentrations, the extraction efficiency of triterpenoids containing polar groups (such as carboxyl and hydroxyl groups) is reduced [29]. In this work, the greatest efficiency in extracting total triterpenoids from PL was observed with a 60% aqueous ethanol solution; thus, this ethanol concentration was selected for further experiments.

3.1.2. Effect of the liquid-solid ratio on the extraction yield

Bioactive compounds in plants can be extracted efficiently with appropriate amounts of solvents. A small amount of solvent relative to the medicinal material will interfere with the mass transfer of target compounds. However, a large amount of solvent will cause unnecessary waste [30]. Therefore, we evaluated the extraction yield of triterpenoids from PL using various liquid-solid ratios: 10, 15, 20, 25 and 30 mL/g (Fig. 1B). The yields gradually increased from 4.00% to 7.22% when the solvent ratio was increased from 10 to 25 mL/g. Subsequently, the yield decreased when the liquid-solid ratio was 30 mL/g. Therefore, a liquid-solid ratio of 25 mL/g was selected for the subsequent experiments.

3.1.3. Effect of time on the extraction yield of total triterpenoids

The tested extraction times were 30, 40, 50, 60, 90 and 120 min (Fig. 1C). The content of triterpenoids increased significantly from 6.09% to 7.31% when the extraction time increased from 30 to 90 min. The maximum extraction yield of total triterpenoids was obtained with an extraction time of 60 min. With longer extraction times, the triterpenoids molecules in the cells dissolved and reached a balance between the inside and outside of the cells. The extraction efficiency increased as the duration of extraction increased. However, with a prolonged extraction time, the extract is mixed with impurities by

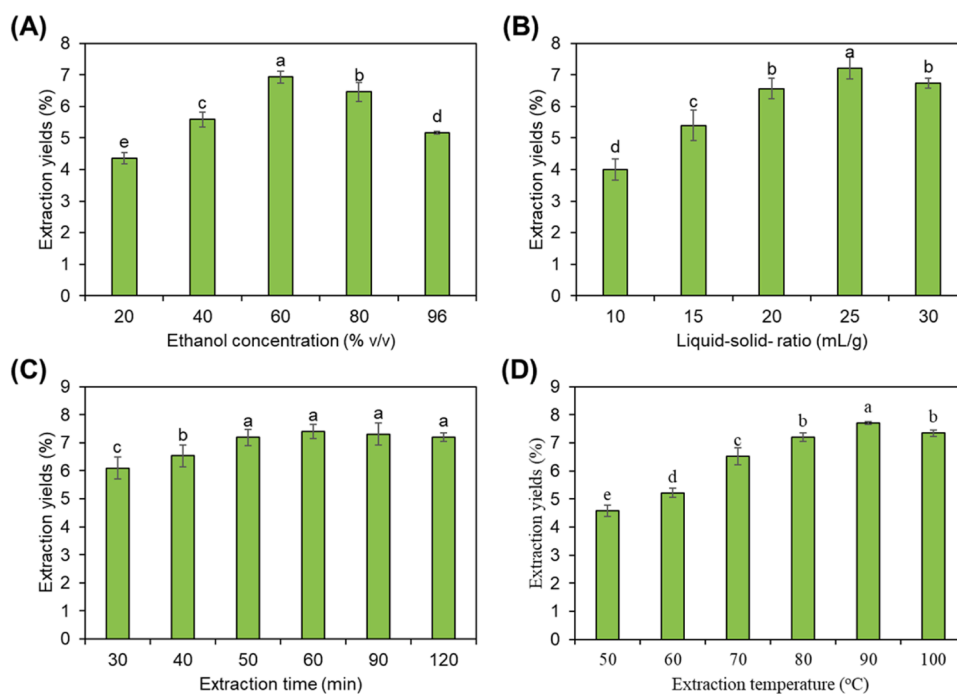


Fig. 1. Effect of different factors on the extraction yields of total triterpenoids from persimmon leaves. A) ethanol concentration, B) liquid-solid ratio, C) extraction time, and D) extraction temperature. Different letters placed above each column indicate significant difference ($p < 0.05$).

increasing the solubility of ethanol-soluble components, which is detrimental to the purification process and lowers the triterpenoids content. Moreover, long extraction and high-temperature extraction may also result in the decomposition of the target compounds [31]. In this study, a 60-minute extraction time was selected.

3.1.4. Effect of temperature on the extraction yield of triterpenoids

Controlling the extraction temperature helps reduce the extraction time and improves extraction efficiency. In this study, extraction temperatures of 50, 60, 70, 80, 90 and 100 °C were evaluated in terms of extraction efficiency (Fig. 1D). An increase in temperature enhances the permeability of triterpenoids molecules to the cell's interior. In the range of 50 to 90 °C, the yield of total triterpenoids increased proportionally with higher temperatures. Raising the temperature results in reduced solvent viscosity, thereby inducing continuous convective diffusion that in turn enhances the solubility and rate of diffusion of a solute in a solvent [32]. However, the yield of total triterpenoids decreased slightly when the temperature exceeded 90 °C because high temperatures may destroy the molecular structure of triterpenoids. Therefore, the optimal extraction temperature to maximise the total yield was 90 °C.

3.1.5. Optimisation of extraction conditions of total triterpenoids by RSM

The goal of optimization is to pinpoint the most suitable solution within the constraints and requirements of the problem. Experimental design involves practical statistical approaches to model and analyze scenarios where the response level is influenced by multiple variables. The RSM is a highly effective optimization method that employs mathematical and statistical techniques to develop experimental models [33]. The BBD is commonly utilized for designing experiments within RSM. Its main advantage lies in addressing the determination of experimental boundaries, particularly to avoid extreme treatment combinations. With only three levels for each factor, BBD is specifically crafted to fit a second-order model, which is the primary focus in most RSM studies [34].

In order to assess the effects and interactions of various factors on the extraction process, we used RSM to optimise the conditions affecting the extraction yield. Experiments for total triterpenoids extraction from PL were designed for optimisation. The experimental design was a Box-Behnken model with 29 experimental units and selected variables. The factors evaluated were the ethanol concentration (A), liquid-solid ratio (B), extraction time (C) and extraction temperature (D). The code-independent variables in Table S1 indicate that the 29 trials produced encoded variables and five central points. The triterpenoids extraction yields (%) is shown in Table S2; the result was the average of triplicate experiments.

The outcomes of the reliability and variance analysis are displayed in Table S3. The values of the variables (A, B, C), the interacting pairs (AB, BC, BD, CD) and the quadratic variables (A^2 , B^2 , C^2) showed statistical significance ($p < 0.05$) when applying the model. The value of Fisher's F-test model (422.57) and the probability value ($p < 0.0001$) (Table S3) demonstrated that the experimental model was set up to show statistical significance. The R^2 represents the percentage of variation in the response variable explained by the model [33]. In this study, an R^2 value of 0.9976 indicates that the proposed model can account for 99.76% of the changes in the extraction efficiency range. The Adjusted R^2 (Adj- R^2) reflects the degree of conformity between the experimental data and the model, taking into consideration the level of model freedom and the number of experiments [33]. A value of Adj- $R^2 = 0.9953$ indicates a strong correlation of 99.53% between the model and experimental data. The Predicted R^2 (Pred- R^2) evaluates the model's predictive capacity, specifically its proficiency in forecasting a given set of new data. The difference between Pred- R^2 and Adj- R^2 is negligible; according to the software default, Pred- R^2 and Adj- R^2 should not differ by more than 0.2 [33].

The quadratic equation correlating the total triterpenoids yield and

the extraction variables was as follows: $y = 8.24 + 0.3969A - 0.4747B + 0.1751C - 0.1942AB + 0.1555BC - 0.0797BD - 0.165CD - 0.9589A^2 - 0.7747B^2 - 0.6553C^2$, where: y is the triterpenoids yield (%), A is the alcohol concentration (%), B is the liquid-solid ratio (mL/g), C is the extraction time and D is the extraction temperature (°C). The equation showed the correlation between the model and the experiment, with $R^2 = 0.9953$ (Fig. 2).

The contour graphs represent the depiction of the interplay among independent variables and the correlation between these independent variables and the dependent variables (Fig. 3). In a broader context, the overall yield of the extraction was influenced by factors such as the ethanol concentration, extraction time and liquid-solid ratio. The extraction efficiency demonstrated a first increase followed by a decrease with increasing levels of both alcohol concentration and the liquid-solid ratio, as well as an increase in the extraction time (Fig. 3A, B, and D). By contrast, the effect of the rise in temperature on the extraction yields was minimal (Fig. 3C, E, and F). These findings align with the outcomes of the variance analysis (Table S3). The p -values for variables A (ethanol concentration), B (liquid-solid ratio) and C (extraction time) were all below 0.0001, whereas the p -value for variable D (extraction temperature) was 0.1037 (>0.05). We also conducted a comprehensive analysis of the interplay between these factors and their combined influence on the total triterpenoids extraction yields. Fig. 3A illustrates the combined impact of the ethanol concentration and liquid-solid ratio, revealing an initial rise and subsequent decline in the extraction yields with an increase in both the ethanol concentration and liquid-solid ratio. A similar pattern is shown in Figs. 3B and 3D, which depict the combined effects of two pairs of factors on the extraction efficiency: ethanol concentration and extraction time, and the liquid-solid ratio and extraction time, respectively. However, the significance of the combined factors on the extraction efficiency varied, with p -values for AB and BC being below 0.0001, whereas the p -value for AC was 0.3668. Fig. 3C illustrates ethanol concentration and extraction temperature's combined impact on extraction efficiency. An increase in the ethanol concentration significantly affected the extraction yield, whereas an increase in the extraction temperature exhibited no notable influence on

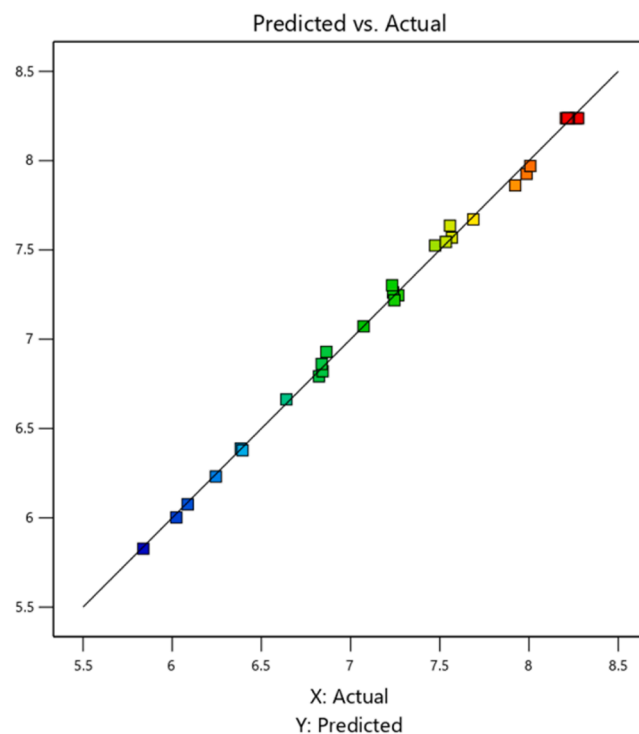


Fig. 2. Correlation between the extraction yields of total triterpenoids from persimmon leaves according to model and experiment.

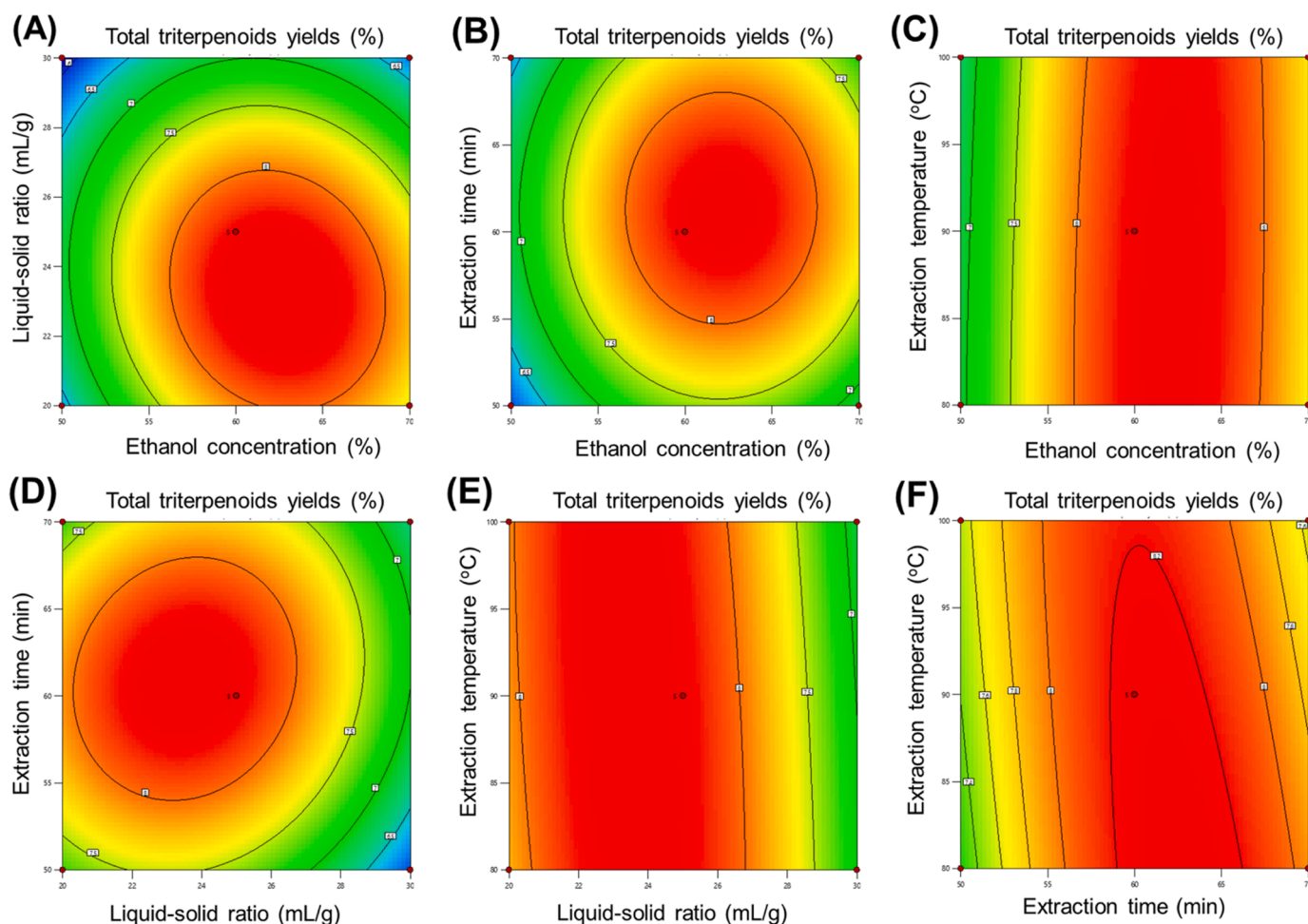


Fig. 3. Graphs illustrating the interactions between different factors in the efficiency of extracting total triterpenoids from persimmon leaves. (i) ethanol concentrations and liquid-solid ratio; (ii) ethanol concentrations and extraction time; (iii) ethanol concentrations and extraction temperature; (iv) liquid-solid ratio and extraction time; (v) liquid-solid ratio and extraction temperature; and (vi) extraction time and extraction temperature.

the extraction efficiency. The same trend persisted for the interaction pairs (extraction temperature and liquid-solid ratio, and extraction temperature and extraction time). Nevertheless, differences emerged in the simultaneous effect of these two factors on the extraction yield. The p -values for CD, BD and AD were < 0.0001 , 0.0073 and 0.1134 , respectively (Table S3).

Using the information from the optimisation procedure, which relied upon the response surface model, the optimal extraction conditions were determined as follows: an ethanol concentration of 63%, a liquid-solid ratio of 23 mL/g, an extraction time of 61 min and an extraction temperature of 82.7 °C. The total triterpenoids yield was expected to be 8.359%. Extracting triterpenoids from PL under the optimal conditions resulted in an experimental total triterpenoids yield of $8.26 \pm 0.30\%$, which was equal to 98.82% of the predicted model. The actual triterpenoids yield obtained under optimal conditions was not significantly deviate from the predicted results ($p > 0.05$). This indicates that the optimal extraction conditions according to the experimental design were consistent with the experimental results. The triterpenoids content in the obtained product was $24.43 \pm 1.26\%$.

Triterpenoids are a group of active ingredients found in various plant species, exhibiting numerous potential biological activities. In the *Sanghuangporus sanghuang* mushroom, the determined total triterpenoid content was 1.33% [29]. Lei et al. reported the total triterpenoid content of 2.67% in *Jatropha curcas* leaves [23]. *Ganoderma lucidum*, widely used in China, South Korea, and Vietnam for its recognized health benefits, is a medicinal mushroom. Triterpenoids constitute the primary active

ingredient group responsible for the biological effects of *Ganoderma lucidum*. Tran et al. utilized the supercritical extraction method to determine the triterpenoid content of Vietnamese *Ganoderma lucidum* as 0.27% [35]. In another study, Yanwei et al. reported the total triterpenoid content of loquat peel (*Eriobotrya japonica*) to be 1.39% [36]. Employing a microwave-based extraction method, the total triterpenoid content in *Lactuca indica* leaves was observed to be 2.92% [37]. Meanwhile, the total triterpenoid content in persimmon leaves, as determined in this study, was 8.26%, which is significantly higher than the levels found in triterpenoid sources from other species.

3.2. Purification of triterpenoids and reuse of solvents

3.2.1. Purification of triterpenoids from the PL total extract

The triterpenoids in the obtained extracts were purified in two stages. First, the water-soluble impurities were removed from the product. Next, different amounts of activated carbon (1, 2, 4, 6, 8 and 10 g) were used to remove chlorophyll and pigments. The results are shown in Fig. 4, where triterpenoids recovery yields are defined as the mass percent of recovered triterpenoids after purification compared to the total mass of triterpenoids present in the original ethanol extract. The purity of triterpenoids was expressed as the mass percent of triterpenoids present in the final product. Triterpenoids recovery yields were inversely proportional to the amount of activated carbon and ranged from 33.11% to 84.11%. By contrast, the purity of the obtained triterpenoids, which ranged from 44.27% to 81.51%, was proportional

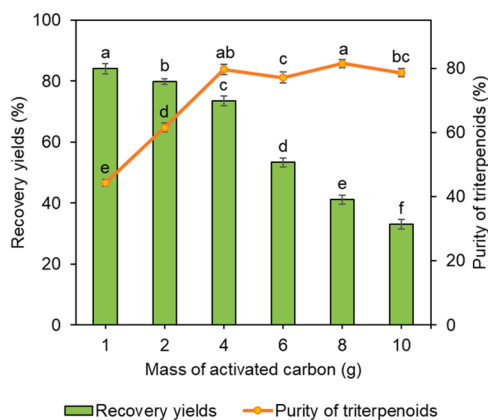


Fig. 4. Recovery yields and purity of triterpenoids of the triterpenoids purification process. The triterpenoids recovery was carried out in two steps: (i) removal of polar impurities with distilled water and (ii) removal of chlorophylls and pigments with activated carbon. In the same graphs type, different letters indicate significant difference ($p < 0.05$).

to the amount of activated carbon. The activated carbon was used to remove chlorophyll and pigments. However, a large amount of adsorbent in the experiment reduced the recovery yields of the target compounds [38]. Therefore, choosing a suitable amount of activated carbon to ensure both the purity of the triterpenoids and the maximum recovery is critical. In this study, the triterpenoids' recovery yields and purity achieved with the ideal quantity of activated carbon (4 g) were 73.57% and 79.66%, correspondingly. Thus, after optimising the purification process, the content of triterpenoids in the obtained product increased from 24.43% to 79.66%.

The HPLC method was employed to validate the purity of the total triterpenoids extract, utilizing ursolic acid, oleanolic acid, diospyric acid B, rotungenic acid, and pomolic acid as reference standards. The relative purity of the total triterpenoids extract was quantified as a percentage of the peak areas of the five standard triterpenoids concerning the total peak areas observed at 210 nm (refer to Fig. 5). The retention times of diospyric acid B, rotungenic acid, ursolic acid, oleanolic acid and pomolic acid were 2.282, 2.785, 3.418, 9.732 and 10.159 min, respectively. The data reveals that the contents of the five triterpenoids accounted for 86.43% of the total triterpenoids, in which the contents of

diospyric acid B, rotungenic acid, ursolic acid, oleanolic acid and pomolic acid were 23.89%, 13.84%, 14.43%, 12.07% and 22.20%, respectively.

3.2.2. Solvent recovery and reuse

Solvent recovery and reuse are necessary for the sustainability of the extraction method. This not only saves money but also limits environmental pollution by the waste solvent. Here, the solvent after the extraction process was evaluated for its reusability. The recovered ethanol was used to extract fresh biomass under optimal conditions, and the triterpenoids were purified with activated carbon. The triterpenoids' extraction yields and purity were assessed in each cycle. Results from three extraction cycles with reused ethanol revealed that the triterpenoids' extraction yields and purity did not change significantly during the extraction process (Fig. 6). This outcome encourages the reuse of

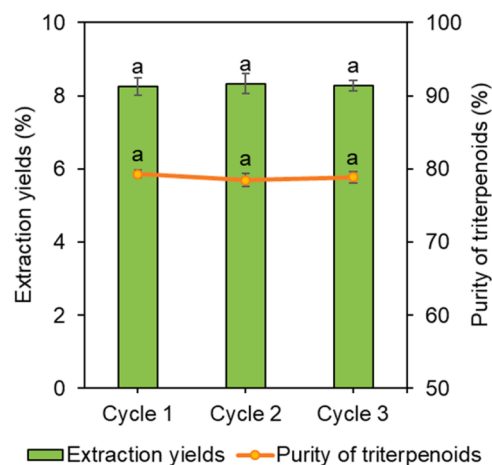


Fig. 6. The triterpenoids' extraction yields and purity were assessed over three extraction cycles with the recycling of ethanol solvent. The extraction was performed under the following conditions: the ethanol concentration of 63%, the liquid-solid ratio of 23 mL/g, the extraction time of 61 min, and the extraction temperature of 82.7 °C. The triterpenoids recovery was carried out in two steps: (i) removal of polar impurities with distilled water and (ii) removal of chlorophylls and pigments with activated carbon. In the same graphs type, different letters indicate significant difference ($p < 0.05$).

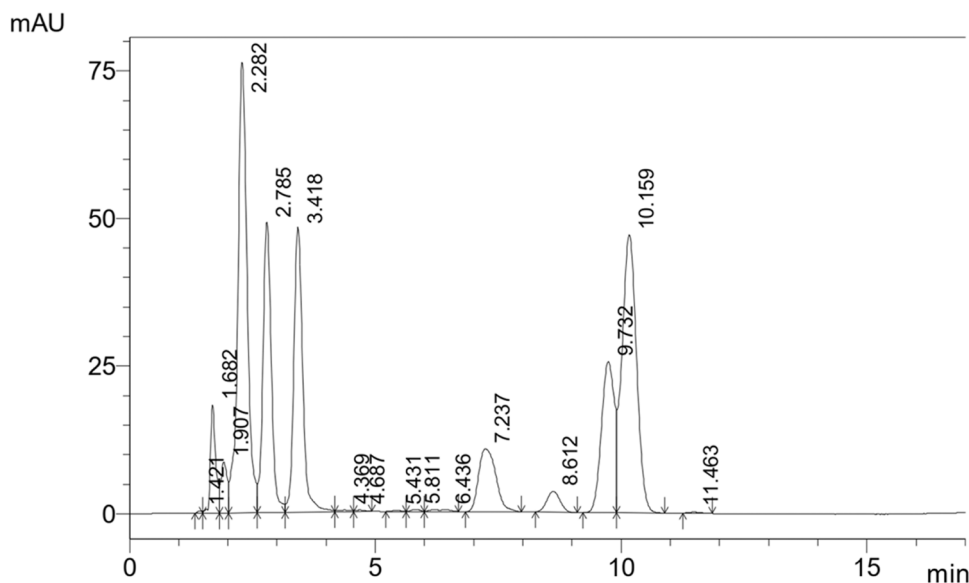


Fig. 5. HPLC chromatogram of the total triterpenoids from persimmon leaves. The analysis process was performed using LC-20AD HPLC with SPD-M20A PDA detector (Shimadzu, Japan).

solvents to improve cost efficiency.

In summary, this study presented a four-stage procedure for the extraction and purification of triterpenoids from PL: (i) a liquid-solid extraction for the total ethanolic extract; (ii) the removal of water-soluble impurities and the recovery of the triterpenoids-rich precipitate; (iii) the removal of chlorophylls and pigments by activated carbon; and (iv) evaporation of the solvent and recovery of triterpenoids by drying (Fig. 7).

3.3. Evaluation of bioactivities of total triterpenoids

3.3.1. Cytotoxic activity

Cancer stands as a leading global cause of mortality. Every year, the number of patients suffering from tumours is approximately 11 million people, and this number is predicted to increase to approximately 13.1 million people by 2030. Unfortunately, most of the clinically available cancer therapies often cause unwanted effects on the patient's body. Thus, multiple studies have focused on developing herbal medicines to treat cancer. Among natural products, triterpenoids exhibit potential bioactivity against cancer cells [39]. In this research, the cytotoxic activity of the total extract and total triterpenoids from PL were evaluated on cancer cell lines (MCF-7, HT-29, SW480, HepG2, SK-LU-1, HeLa and AGS) and normal cells (HEK-293). The results presented in Table 1 show that the total triterpenoids had cytotoxic activity against the studied cell lines, with IC_{50} values from 32.49 to 55.02 $\mu\text{g/mL}$. Nonetheless, the total extract could not inhibit the growth of these cell lines. This result proves that the degree of growth inhibition is proportional to the content of triterpenoids in the products. The contents of total triterpenoids in the total extract and purified extract were 24.43% and 78.07%, respectively. Some studies have indicated that PL extracts may destroy cancer cells. Kim et al. reported that the ethanolic extract of PL had a cytotoxic effect on HepG2 and HEK-293A cells. The number of HepG2 cells was significantly reduced at a dose of 50 $\mu\text{g/mL}$ but this was not observed with the HEK-293A cell line [40]. According to Jo et al., methanolic extracts of the persimmon calyx exhibited inhibitory activity against HeLa, HT-29 and PANC-1 (human pancreatic cancer) cells [41]. Inhibition of nitrosamine-induced squamous epithelial cell proliferation and cancer in rats was observed during treatment in vivo with PL. In another in vivo experiment, PL exhibited anti-tumour effects on S-180 (murine sarcoma

Table 1

Cytotoxic activity of total extract and total triterpenoids from persimmon leaves.

Cell lines	IC_{50} ($\mu\text{g/mL}$)		
	Total extract	Total triterpenoids	Ellipticine
MCF-7	>100	55.02 \pm 2.92	0.32 \pm 0.02
HT-29	>100	32.49 \pm 1.39	0.42 \pm 0.03
SW480	>100	41.56 \pm 3.92	0.42 \pm 0.04
HepG2	>100	41.95 \pm 2.66	0.45 \pm 0.03
SK-LU-1	>100	37.09 \pm 3.22	0.51 \pm 0.04
HeLa	>100	48.91 \pm 2.30	0.47 \pm 0.05
AGS	>100	42.36 \pm 3.87	0.39 \pm 0.03
HEK-293A	>100	44.75 \pm 3.09	0.30 \pm 0.02

Data are the mean of three replicates \pm standard deviation.

HepG2: human hepatocellular carcinoma cell line; MCF-7: human breast carcinoma cells cell line; HT-29: human colon carcinoma cell line; SW480: human colonic carcinoma cell line; SK-LU-1: lung carcinoma cell line; HeLa: human cervical carcinoma cell line; AGS: human gastric carcinoma cell line; HEK-293A: human embryonic kidney cell line; total extract: ethanol extract of persimmon leaves; and total triterpenoids: triterpenoids purified from total extract.

cancer cell line) [18]. The results of this study demonstrated that triterpenoids extracted from PL are potential candidates for the development of anti-cancer agents.

3.3.2. α -Glucosidase inhibitory activity

Diabetes causes serious consequences for the patient's body. In recent decades, there has been a significant surge in the prevalence of diabetes. Projections indicate that by 2030, the global diabetic population will reach 552 million individuals [42]. Several drugs that inhibit the α -glucosidase enzyme, including acarbose, voglibose, miglitol and 1-deoxynojirimycin, have been used to treat diabetes. However, these drugs are known to cause serious gastrointestinal side effects [43]. In the current study, we evaluated the α -glucosidase inhibition effect of the total extract and total triterpenoids derived from PL. The samples exhibited much stronger α -glucosidase inhibitory activity than acarbose as a positive control. The IC_{50} values of the total extract, total triterpenoids and acarbose were 0.18 \pm 0.05, 2.12 \pm 0.15 and 143.44 \pm 2.5 $\mu\text{g/mL}$, respectively (Table 2). Several studies have reported the antidiabetic activity of extracts and compounds derived from PL. Bae

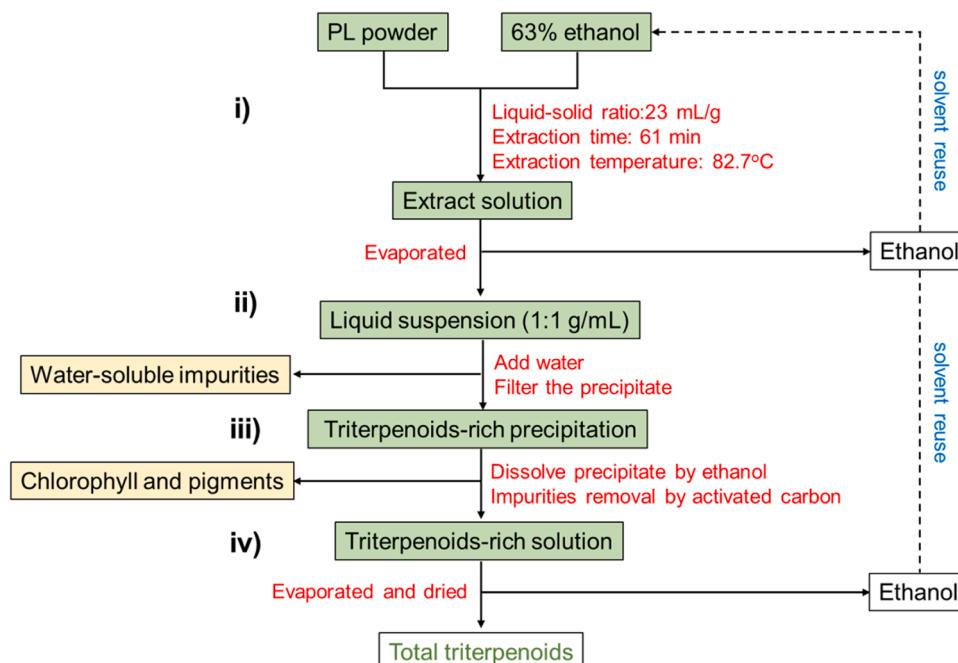


Fig. 7. The schematic diagram of the extraction and purification process of triterpenoids from persimmon leaves was proposed in this study.

Table 2
Biological activities of total extract and total triterpenoids from from persimmon leaves.

Biological activities	IC ₅₀ (µg/mL)			
	α-Glucosidase inhibitory	Antioxidant	Anti-inflammatory	AChE inhibitory
Total extract	0.18 ± 0.05	12.53 ± 0.20	>100	112.22 ± 9.68
Total triterpenoids	2.12 ± 0.15	13.92 ± 0.18	29.55 ± 1.67	40.07 ± 2.54
Quercetin	-	6.29 ± 0.11	-	-
Galantamine	-	-	-	0.33 ± 0.01
L-NMMA	-	-	8.92 ± 0.85	-
Acarbose	143.44 ± 2.5	-	-	-

Data are the mean of three replicates ± standard deviation.

Total extract: ethanol extract of persimmon leaves; and total triterpenoids: triterpenoids purified from total extract.

et al. demonstrated the anti-diabetic effect of the aqueous extract of PL through the inhibition of α-glucosidase and the protection of pancreatic β-cells in *db/db* mice and streptozotocin-induced diabetic mice [44]. In another report, the PL extract showed anti-diabetic effects in normal and diabetic rats by obstructing carbohydrate hydrolysis and glucose absorption in the intestine [45]. Adding PL to the normal diet of type 2 diabetic mice substantially ameliorated hyperglycaemia and hyperlipidaemia [46]. Therefore, the extracts and triterpenoids from PL have great potential for the development of an anti-diabetic drug.

3.3.3. Antioxidant activity

The compounds and extracts from PL have long been known for their potent antioxidant activities. In this study, the DPPH inhibitory tests of the total extract and total triterpenoids showed that the antioxidant activities of the extracts were remarkable (Table 2B). The IC₅₀ values of the total extract, total triterpenoids and quercetin were 12.53 ± 0.20, 13.92 ± 0.18 and 6.29 ± 0.11 µg/mL, respectively. Several studies have demonstrated that PL has free-radical-scavenging activity. Research by Han et al. [47] reported a low IC₅₀ value (0.11 mg/mL) for the methanol extract of PL against the DPPH radical. Abul et al. [48] reported that aqueous extracts were capable of scavenging DPPH and ABTS. According to a study conducted in 2002 by Chen et al. [49], five triterpenoids derived from PL (ursolic acid, α-amyrin, uvaol, 19 α-hydroxy ursolic acid and 19 α, 24-dihydroxy ursolic acid) substantially prevented the generation of superoxide by N-formyl-methionyl-leucyl-phenylalanine and arachidonic acid. In another study, Jaeyoung et al. screened the antioxidant activity of the ethanol extract, ethyl acetate fraction, water fraction and 27 isolated compounds from PL [20]. In addition, various classes of non-triterpenoids derived from PL have also been reported to have antioxidant activity. Total flavonoids from PL exhibited scavenging activity of the DPPH radical, hydroxyl radical and superoxide anion, as well as metal-chelating activities and reducing power [50]. The results of this experiment once again confirmed the potent antioxidant activity of PL.

3.3.4. Anti-inflammatory activity

In this study, nitric oxide (NO) inhibition by the total extract and triterpenoids from PL was evaluated in vitro and is presented in Table 2. The total extract did not inhibit NO production, whereas total triterpenoids notably inhibited NO production in LPS-activated RAW 264.7 macrophages (IC₅₀: 29.55 ± 1.67 µg/mL). Abdelaaty et al. [51] tested the anti-inflammatory activities of ethyl acetate, butanol and the aqueous fractions from PL on Wistar rats and Swiss albino mice. As a result, butanol and the aqueous fractions exhibited anti-inflammatory properties. A study in 2016 isolated two triterpenoids compounds (coussaric acid and betulinic acid) from PL extracts. These two compounds exhibited anti-inflammatory activity on RAW 264.7 cells by inhibiting NO production. In addition, they suppressed inflammatory mediators through the inhibition of NF-κB pathways in RAW 264.7 macrophages stimulated by LPS [52]. Kim et al. demonstrated the role of the ethanol extract of PL in preventing and treating dry-eye disease. The ethanol extract of PL effectively enhanced the conjunctival goblet cell

viability and diminished the inflammatory feedback [53]. Saud et al. reported the anti-inflammatory effect of the chloroform fraction from PL on carrageenan-induced and histamine-induced paw oedema in mice at a dose of 200 mg/kg, which reduced inflammation by 73.87% and 61.22%, respectively [54]. Our results contribute to understanding the anti-inflammatory activity of triterpenoids from PL and support the use of PL as an herbal remedy.

3.3.5. AChE inhibitory activity

AChE catalyses the conversion of acetylcholine into choline and acetate in the synaptic cleft. In Alzheimer's patients, AChE inhibition leads to improved cognitive function. The AChE inhibitory activities of the astragalus and triterpenoids from PL are displayed in Table 2. The findings indicated that the inhibitory effect on AChE by total triterpenoids exceeded that of the total extract, with IC₅₀ values of 40.07 ± 2.54 and 112.22 ± 9.68 µg/mL, respectively. This result confirms the role of triterpenoids on AChE inhibitory activity. According to Nguyen et al. [55], 12 serratene-type triterpenoids from *Lycopodiella cernua* (a traditional Chinese herb) were isolated and structurally determined, among which 3β,21α-diacetoxyserratane-14β-ol showed the strongest AChE inhibitory activity with an IC₅₀ value of 0.22 µM. Evelyn et al. [56] evaluated the AChE inhibitory activity of five ceanothane triterpenoids derived from Chilean Rhamnaceae and their two semi-synthetic derivatives. The results showed that a 1,3-didehydro derivative of ceanothic acid exhibited the highest AChE inhibitory activity (IC₅₀: 0.126 µM). In another report by María et al. [14], seven triterpenoids (calenduladiol, lupeol, heliantriol B2, faradiol, taraxasterol, pseudotaraxasterol, α-amyrin and β-amyrin) were isolated from the ethanolic extract of *Chuquiraga erinacea*. Among them, calenduladiol and its semisynthetic derivative disodium calenduladiol disulfate indicated the highest AChE inhibitory activities (31.2% and 94.1%, respectively, at 0.5 mM). Triterpenoids alkaloids from *Buxus papillosa* were isolated and evaluated for their AChE inhibitory activity and indicated IC₅₀ values between 25.4 and 235 µM [57]. Moreover, the ethyl acetate fraction from persimmon exhibited neuroprotective effects in an immortalised mouse hippocampal cell line (HT22) and ameliorated cognitive dysfunction in trimethyltin chloride-induced mice [58]. Therefore, our results are consistent with previous reports on the beneficial effects of PL on the nervous system.

3.3.6. Antimicrobial activity

The antimicrobial activity of the total extract and total triterpenoids were evaluated against seven bacterial and fungal strains: the Gram-positive *S. aureus*, *B. subtilis* and *L. fermentum*, the Gram-negative *S. enterica*, *E. coli* and *P. aeruginosa*, and the *C. albicans* fungus. As shown in Table 3, neither the total extract nor total triterpenoids exhibited inhibitory activity against bacteria or fungi (IC₅₀ > 256 µg/mL).

4. Conclusions

In this work, for the first time, we provide information on a

Table 3

Antimicrobial activities of total extract and total triterpenoids from persimmon leaves.

Bacterial/ Fungal	IC ₅₀ (µg/mL)				
	Total extract	Total triterpenoids	Ampicillin	Cefotaxime	Nystatin
<i>S. aureus</i>	>256	>256	0.02 ± 0.005	-	-
<i>B. subtilis</i>	>256	>256	3.62 ± 0.15	-	-
<i>L. fermentum</i>	>256	>256	1.03 ± 0.07	-	-
<i>S. enterica</i>	>256	>256	-	0.43 ± 0.05	-
<i>E. coli</i>	>256	>256	-	0.007 ± 0.002	-
<i>P. aeruginosa</i>	>256	>256	-	4.34 ± 0.15	-
<i>C. albican</i>	>256	>256	-	-	1.32 ± 0.05

Data are the mean of three replicates ± standard deviation.

Total extract: ethanol extract of persimmon leaves; and total triterpenoids: triterpenoids purified from total extract.

triterpenoids-rich medicinal plant, persimmon leaves. The extraction yield was found under optimal conditions to be 8.26%. The content of triterpenoids in the obtained product was upgraded from 24.43% to 79.66% by using activated carbon for the purification process with a triterpenoids recovery efficiency of 73.57%. The ethanol solvent was efficiently reclaimed and reused for at least three cycles to extract triterpenoids from PL, without altering either the extraction yield or the purity of the obtained triterpenoids. The total extract and triterpenoids demonstrated a promising α -glucosidase inhibitory activity. Total triterpenoids also exhibited activity against cancer cells (MCF-7, HT-29, SW480, HepG2, SK-LU-1, Hela, AGS), and anti-inflammatory activity. Both total extract and triterpenoids also exhibited strong antioxidant activity. Triterpenoids showed higher inhibition of AChE inhibition compared with the total extract, meanwhile, they did not show antimicrobial activity on the studied bacteria and fungi strains. In conclusion, the results suggest a source of medicinal herbs rich in triterpenoids. The extraction and purification process proposed in this work was simple, and efficient, and was expected to be carried out on an industrial scale. The bioactivities of the triterpenoids show potentials for their applications in drug development.

CRediT authorship contribution statement

Hung Van Nguyen: Conceptualization, Methodology, Investigation, Writing – original draft preparation. **Nhan Trong Le:** Conceptualization, Methodology, Investigation, Writing – original draft preparation. **Nguyen Thao Nguyen Le:** Investigation, Writing – original draft preparation: investigation, formal analysis. **Thu Dong Duong:** Investigation. **Trinh Thuc Le:** investigation. **Ha Thu Thi Nguyen:** Methodology, Investigation. **Huong Thanh Phung:** Writing – review and editing, Supervision. **Hoai Thi Nguyen:** Conceptualization, Writing – review and editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.procbio.2024.01.025](https://doi.org/10.1016/j.procbio.2024.01.025).

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