Assessment of the $\alpha$-glucosidase and $\alpha$-amylase inhibitory potential of *Paliurus spina-christi* Mill. and its terpenic compounds

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Abstract
Type II diabetes mellitus is a common disease in the world and characterized by hyperglycemia. Prevention of diabetes by reducing hyperglycemia depends on the inhibition of $\alpha$-amylase and $\alpha$-glucosidase enzymes. In this study, the antidiabetic profiles of the *Paliurus spina-christi* Mill. fruit were investigated. The fruit of this species is used as an antidiabetic in folk medicine in Turkey. $\alpha$-amylase and $\alpha$-glucosidase inhibitory effect studies were conducted to prove this effect. The $n$-hexane sub-extract of the methanolic fruit extract (IC$_{50}$ = 445.7 ± 8.5 µg/mL) showed greater inhibitory activity against $\alpha$-glucosidase than acarbose (IC$_{50}$ = 4212.6 ± 130.0 µg/mL), in contrast to its slight/no inhibitory effect on $\alpha$-amylase. The phytochemical investigation of the $n$-hexane sub-extract of the *P. spina-christi* fruit led to the isolation of three triterpenes, namely betulin (1a), betulinic acid (1b), and lupeol (2), and a sterol ($\beta$-sitosterol) (3). The structures of compounds 1-3 were further analyzed using extensive 1D- and 2D-NMR, and the results were compared with literature. Betulin (1a), betulinic acid (1b), and lupeol (2) are reported from this species for the first time. All the isolated compounds, especially betulin (1a) and betulinic acid (1b) mixture (IC$_{50}$ = 248 ± 12 µM) showed higher $\alpha$-glucosidase inhibitory activity than acarbose (IC$_{50}$ = 6561 ± 207 µM). As extracts, the compounds were also found to be ineffective against $\alpha$-amylase.

Graphical abstract

Keywords *Paliurus spina-christi* Mill. · Betulin · Betulinic acid · Lupeol · $\beta$-sitosterol · $\alpha$-Glucosidase inhibition

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**Introduction**

Diabetes mellitus (DM) is an endocrine disease characterized by hyperglycemia and associated with deficiency or excess in insulin secretion resulting from irregularities in carbohydrate, fat, and protein metabolism. Retinopathy, nephropathy, neuropathy, microangiopathy, and cardiovascular disease risks may arise as a result of long-term diabetes. Type II DM is known as non-insulin-dependent diabetes which accounts for 90–95% of all diabetes cases [1]. There are several groups of antidiabetic drugs to treat patients with type II DM, including sulfonylureas, meglitinide/phenylalanine analogs, dipeptidyl peptidase-4 inhibitors, biguanides, thiazolidinediones, α-glucosidase inhibitors, sod-glucose cotransport-2 inhibitors, and dopamine D2 agonists [2, 3] [Fig. 1]. α-glucosidase inhibitors slow down carbohydrate digestion and decrease the postprandial rise in the levels of blood glucose. Due to the high cost and side effects of synthetic development due to their chemical diversity. From this point of view, we hypothesized that the discovery of selective α-glucosidase inhibitors rather than agents inhibiting both α-amylase and α-glucosidase could be of great importance to identify more reliable and less toxic drug candidates for type II DM. The antihyperglycemic effects of *P. spina-christi* fruit extracts prepared in different concentrations using solvents of different polarities and pure compounds isolated from the fruit were evaluated as an indicator of their antidiabetic potential. Seventy percent of the methanolic extract was shown to decrease blood sugar and glycated hemoglobin levels in rats with diabetes induced by streptozotocin [11]. The plant contains different classes of phytochemicals, such as terpenes, flavonoids, tannins, amino acids, alkaloids, and sterols [8, 9], which may be responsible for the therapeutic effects of the plant. Thus, the phytochemical ingredient may be of vital importance.

One of the aims of our study was to isolate the secondary metabolites of the *P. spina-christi* fruit and analyze their structures. Second, we aimed to investigate the antidiabetic potential of various extracts of the *P. spina-christi* fruit and its compounds we isolated by examining their enzyme inhibitory effects. The antidiabetic properties of the plant were evaluated using α-glucosidase and α-amylase inhibitory studies. The methanolic fruit extract and its sub-extracts (70% methanol, *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water) and isolated compounds were tested to obtain evidence of the traditional use of the *P. spina-christi* fruit as an antidiabetic agent and evaluate the antidiabetic properties of its phytochemical components in terms of enzyme inhibitory effects.

**Results and discussion**

Following the ethnobotanical investigation of natural products, it is necessary to conduct biological activity studies to provide evidence for their known activities. Plant phyto-components play a role in the emergence of the effects of plants. Natural products, standardized extracts, or pure compounds have significant potential for new drug development due to their chemical diversity. From this point of view, we hypothesized that the discovery of selective α-glucosidase inhibitors rather than agents inhibiting both α-amylase and α-glucosidase could be of great importance to identify more reliable and less toxic drug candidates for type II DM. The antihyperglycemic effects of *P. spina-christi* fruit extracts prepared in different concentrations using solvents of different polarities and pure compounds isolated from the fruit were evaluated as an indicator of their antidiabetic potential. Seventy percent of the methanolic extract was prepared from the fruit, and its sub-extracts were fractionated with solvents of different polarity ranging from non-polar to polar, such as *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water. The reason why we investigated various extracts prepared with solvents of different polarity is that each extract contains compounds suitable for its polarity, and therefore their phytochemical composition differs according to the polarity of the solvent from which they are extracted.

In particular, 70% methanolic extract (IC$_{50}$ = 529.2 ± 38.6 µg/mL) and *n*-hexane sub-extract (IC$_{50}$ = 445.7 ± 8.5 µg/mL) considerably inhibited α-glucosidase, indicating that they may have significant potential, and they also showed higher activity than acarbose (IC$_{50}$ = 4212.6 ± 130.0 µg/mL) (Table 1). The highest effect was detected in the *n*-hexane sub extract; therefore, isolation studies were carried out on this extract.

A total of four compounds were isolated. There were three triterpenes [betulin (1a) and betulinic acid (1b) in a mixture], and lupeol (2), and one sterol (β-sitostanol (3)}
The structures of the isolated compounds were identified using spectral methods, namely 1D (1H-NMR, 13C-NMR and DEPT), 2D (COSY, HMQC, and HMBC), and HRESIMS, and the results were compared with the literature.

Betulin (1a), betulinic acid (1b), and lupeol (2) were isolated from this species for the first time, in addition to betulin and lupeol were isolated from genus *Paliurus* for the first time. According to previous studies, betulinic acid has been isolated from *Paliurus hemsleyanus* [12], *P. ramossissimus* [13], as well as betulin and lupeol have been isolated from the genus *Ziziphus*, taxonomically belonging to the Paliureae tribe as *Paliurus* [14–17].

In the current study, lupeol, betulin, betulinic acid, and β-sitosterol were determined to significantly inhibit α-glucosidase. Acarbose was used as the positive control.

### Table 1 α-Glucosidase inhibitory activities of the extracts

| Extract   | IC₅₀ value (µg/mL) | Mean        | Standard deviation | Median | Minimum | Maximum | Chi-square | P     | Post-hoc          |
|-----------|--------------------|-------------|--------------------|--------|---------|---------|------------|-------|-------------------|
| 70% methanol | 529.2              | 38.6        |                    | 530.8  | 490.6   | 567.8   | 19.636     | 0.003 | n-hexane sub-extract-Acarbose |
| n-Hexane  | 445.7              | 8.5         |                    | 447.8  | 436.1   | 452.6   |            |       |                   |
| Chloroform| 797.1              | 27.0        |                    | 794.2  | 772.5   | 826.1   |            |       |                   |
| Ethyl acetate | 3921.9            | 28.0        |                    | 3896.4 | 3893.4  | 3943.4  |            |       |                   |
| n-Butanol | 1056.1             | 176.5       |                    | 1001.9 | 930.2   | 1265.3  |            |       |                   |
| Water     | 693.9              | 21.7        |                    | 694.7  | 674.5   | 717.8   |            |       |                   |
| Acarbose* | 4212.6             | 130.0       |                    | 4222.8 | 4078.2  | 4337.7  |            |       |                   |

*aPositive control for α-glucosidase inhibitory activity*
(IC$_{50}$ = 6561 ± 207 µM). The betulin (1a) and betulinic acid (1b) mixture showed the highest activity (IC$_{50}$ = 248 ± 12 µM). The remaining compounds, lupeol (2) (IC$_{50}$ = 2585 ± 79 µM) and $\beta$-sitosterol (3) (IC$_{50}$ = 2939 ± 76 µM), were also found to be more active than acarbose (Table 2).

The strong $\alpha$-glucosidase inhibitory activity of pentacyclic triterpenes can be attributed to the presence of functional groups (COOH and CH$_2$OH) at the C-28 position of the molecule [18]. When all the extracts and compounds were evaluated for their $\alpha$-amylase inhibitory activities, none was found to be effective against the enzyme compared with the acarbose as the positive control (63.7%, Table 2). The strong $\alpha$-glucosidase inhibitory activity of pentacyclic triterpenes can be considered as new therapeutic candidates in the treatment of type II DM.

### Table 2 $\alpha$-Glucosidase inhibitory activities of the isolated compounds

| Compound                  | IC$_{50}$ value (µM) | Mean     | Standard deviation | Median | Minimum | Maximum | Chi-square | P      | Post-hoc |
|---------------------------|----------------------|----------|--------------------|--------|---------|---------|------------|--------|----------|
| Betulin (1a) and Betulinic acid (1b) | 248 ± 12 | 257      | 237                | 259    | 10.385  | 0.016   | 1a, 1b, and acarbose |
| Lupeol (2)                | 2585 ± 79           | 2523     | 2516               | 2657   |         |         |            |        |          |
| $\beta$-sitosterol (3)    | 2939 ± 76           | 2911     | 2857               | 2965   |         |         |            |        |          |
| Acarbose*                 | 6561 ± 207          | 6577     | 6315               | 6723   |         |         |            |        |          |

*Positive control for $\alpha$-glucosidase inhibitory activity

In this study, we examined the antidiabetic properties of the $P$. spina-christi fruit extracts and their phytoconstituents to form a basis for the ethnobotanical use of this species in folk medicine. To the best of our knowledge, this is the first study to isolate potent compounds responsible for the $\alpha$-glucosidase inhibitory effect of the $P$. spina-christi fruit. Three triterpenes, namely betulin (1a), betulinic acid (1b), lupeol (2) and one sterol, $\beta$-sitosterol (3), was isolated. Compounds 1a, 1b, and 2 were isolated from this species for the first time. The betulin (1a) and betulinic acid (1b) isolated from n-hexane sub-extract was determined to significantly inhibit $\alpha$-glucosidase. The herbal remedies prepared from $P$. spina christi fruit and isolated compounds can be considered as new therapeutic candidates in the treatment of type II DM.

### Conclusion

### Experimental

#### General experimental procedures

1D ($^1$H-NMR, $^{13}$C-NMR and DEPT) and 2D (COSY, HMQC and HMBC) NMR spectra were measured on the Varian Mercury Plus (400 MHz for $^1$H-NMR and 100 MHz for $^{13}$C-NMR) spectrometer with TMS as an internal standard at Atatürk University Faculty of Science. HRESIMS data were recorded on an Agilent 6530 Accurate-Mass apparatus and AB Sciex TripleTOF 4600 at the East Anatolia High Technology Application and Research Center of the same university. UV spectra were obtained with a Thermo Scientific Multiskan Go UV-Vis spectrometer. Open column chromatography (CC) was carried out on silica gel 60 (0.063–0.2 mm) (Merck, Germany). The solvents were purchased from Sigma-Aldrich (USA). TLC analyses were performed on precoated silica gel 60 F$_{254}$ plates (Merck), and the spots were visualized by spraying with 1% solution of vanillin in concentrated sulfuric acid followed by heating at 110°C. Dinitrosalicylic acid, $\alpha$-glucosidase enzyme (from Saccharomyces cerevisiae) (EC 3.2.1.20), $\alpha$-amylase enzyme (from pig pancreas) (EC...
3.2.1.1), acarbose, and p-nitrophenol-α-D-glucopyranoside
used in the bioactivity assays were purchased from Sigma-
Aldrich (USA).

Plant material

P. spina-christi Mill. fruit was collected from Uzundere
town located in Erzurum province, Turkey in July 2016.
The plant material was authenticated by forest engineer
Mehmet ÖNAL, MSc from the Eastern Anatolia Forestry
Research Institute. A voucher specimen (No. AUEF 1348)
was deposited at the Biodiversity Application and Research
Center of Atatürk University, Erzurum, Turkey.

P. spina-christi branches with fruit were dried under
shade at room temperature until completely dried. After
removing the soil, the fruit was separated from the branches
and pulverized using a mechanical blender. The dried
samples were then extracted using solvents of different
polarity.

Extraction and isolation of pure compounds

P. spina-christi Mill. dry fruit (800 g) was powdered and
left to maceration overnight with 2 liters of 70% methanol,
and then extracted three times for three hours at 40 °C using
a mantle heater and reflux cooler. Seventy percent methanol
was preferred because it has a polarity close to traditional
usage and is suitable for the extraction of all metabolites,
both polar and non-polar. The filtered extracts were con-
centrated to dryness in the rotary evaporator at 40 °C and
120 rpm. The methanol extract (33.6 g) in water was frac-
tionated using

\[\text{n-butanol} \rightarrow \text{ethyl acetate} \rightarrow \text{n-hexane} \rightarrow \text{chloroform} \rightarrow \text{ethyl acetate} \rightarrow \text{n-butanol} \rightarrow \text{α-hexane}\]

The methanol extract (33.6 g) in water was fractionated using
n-hexane (4 × 500 mL), chloroform (4 × 500 mL), ethyl acetate (6 × 500 mL), and n-butanol (9 × 500 mL) in that order. The sub-extracts and the remaining water phase were concentrated. At the end of the condensation process, 2.2 g n-hexane, 4.1 g chloroform, 2.5 g ethyl acetate, 10.2 g n-butanol, and 11.2 g water sub-extracts were obtained. Isolation studies were performed on the n-hexane sub-extract since it showed the highest α-glucosidase inhibitory effect.

The n-hexane sub-extract (2.18 g) was fractionated by silicone gel CC (70-230 mesh) with n-hexane:ethyl acetate (100:0 → 0:100, v/v) to yield six fractions. Fr. A-F. Fr. B (88.5 mg), Fr. D (161.5 mg), and Fr. F (303.4 mg) were crystallized to obtain compound 2 (19.5 mg), compound 3 (7.4 mg), and a mixture of compounds 1a and 1b (60.8 mg, respectively).

Betulin (1a) and betulinic acid (1b): Amorphous white powder. HRESIMS m/z 425.38021 [(M-OH)+ C30H50O2; calcd. 425.3811] and 455.35860 (M-H)+ C30H48O3; calcd. 456.3603)]. 1H NMR and 13C NMR data were identical to those reported in the literature (Table S1) [23, 24, Figs. S1–S7].

Lupeol (Lup-20(29)-en-3β-ol) (2): Isolated as a white amorphous powder. Its molecular formula, C30H50O, was determined by positive HRESIMS ion observed at m/z 409.38285 (M-OH)+ (calcd. 426.3862). 1H NMR and 13C NMR data were consistent with the literature (Table S1) [25, Figs. S8–S13].

β-sitosterol [(24 R)-24Etalkolest-5-en-3β-ol] (3): Isolated as a white amorphous powder. Its molecular formula, C29H49O, was determined by positive HRESIMS ion observed at m/z 414 (M+) (calcd. 414.384). 1H NMR and 13C NMR data were in agreement with the literature (Table S2) [26, Figs. S14–S19].

α-glucosidase enzyme inhibitory assay

The α-glucosidase inhibitory effect was determined as previously described [27] with slight modifications [28]. All the samples (20 μL), enzyme solution (10 μL, 1 Unite/mL), and potassium phosphate buffer (50 μL, 50 mM, pH 6.9) were mixed in the plate. The mixture was incubated at 37 °C for 5 min. Then, as a substrate, p-nitrophenyl-α-D-glucopyranoside (20 μL, 3 mM) was added for the initiation of reaction, and the mixture was incubated at 37 °C for 30 min. After incubation, 0.1 M sodium carbonate (50 μL) was added to all the wells to quench the reaction. All the solutions were prepared in a buffer system. Acarbose was used as a positive control. The amount of released p-nitrophenol was measured using a 96-well microplate reader at 405 nm. Each assay for all the samples was performed in triplicate. The results were given as the percentage inhibition of enzyme activity and the IC50 value. The percentage inhibition of all the samples was calculated using the equation below:

\[\text{Inhibition}(\%) = \left(1 - \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{control}}} \right) \times 100\]

α-amylase enzyme inhibitory assay

The α-amylase inhibitory effect was determined as previously described [29] with slight modifications [28]. All the samples (100 mL) and 1% starch solution (100 mL) in 20 mM sodium phosphate buffer (pH 6.9 with 6 mM sodium chloride) were incubated in a 24-well microplate at 25 °C for 10 min. After incubation, 100 μL α-amylase solution (0.5 mg/mL) was added to each well, and the reaction mixtures were incubated at 25 °C for 10 min. After incubation, the dinitrosalicly acid color reagent (200 μL) was added to stop reaction. The microplate was then incubated in a boiling water bath for 5 min and cooled to room temperature. From each well, 50 μL was taken and added to a 96-well microplate. The reaction mixture was diluted with 200 μL distilled water, and absorbance was measured at 540 nm. Acarbose was used as a positive control. Each
Inhibition(%) = \left(1 - \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{control}}} \right) \times 100

**Statistical analysis**

All the experiments were performed in triplicate. The Kruskal-Wallis test was used to determine statistical significance. The results were analyzed using SPSS (IBM SPSS Statistics 20, IBM Corporation, Armonk, NY, USA) at the significance level of \( P = 0.05 \). The percentage inhibition and IC50 value data for the extracts and compounds were presented as mean ± standard deviation values.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare no competing interests.

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