Assessment of α-glucosidase and α-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 *α*-amylase and *α*-glucosidase enzymes. In this study, antidiabetic profiles of *Paliurus spina-christi* Mill. phytochemicals and extracts were investigated. The plant is used in folk medicine in Turkey as antidiabetic. *α*-Amylase and *α*-glucosidase inhibitory effect studies were conducted to prove this effect. The *n*-hexane extract (IC_{50} = 445.7 ± 8.5 µg/mL) possessed potent inhibitory activity against *α*-glucosidase than that of acarbose (IC_{50} = 4212.6 ± 130.0 µg/mL), unlike their slight/no inhibition on *α*-amylase. Phytochemical investigation of the *n*-hexane extract of the fruits of *P. spina-christi* led to the isolation of triterpenes, betulin **(1a)**, betulinic acid **(1b)**, lupeol **(2)** and a sterol, *β*-sitosterol **(3)**. The structures of compounds **1–3** were elucidated by extensive 1D- and 2D-NMR and comparison with literature. Betulin **(1a)**, betulinic acid **(1b)** and lupeol **(2)** are reported for the first time from this species. All isolated compounds, especially betulin (**1a**) and betulinic acid (**1b**) mixture (IC_{50} = 248 ± 12 µM) showed higher *α*-glucosidase inhibitory activity than acarbose (IC_{50} = 6561 ± 207 µM). As extracts, the compounds were also found to be ineffective against *α*-amylase.

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 people with diabetes [1]. *α*-Glucosidase inhibitors used in treatment of type II DM, slow carbohydrate digestion, and decrease postprandial rise in the levels of blood glucose. Due to huge prices and side effects related to synthetic medications, there is rising to use natural drugs, where phytochemicals function beneficially [2].

P. spina-christi Mill. belongs to the family known as the Rhamnaceae. It is grown naturally in South of Europe, Turkey, Crimea, the Caucasus, Syria, Iran and Iraq [3, 4]. This species is 2–4 m high, very prickly shrub. The fruits of the plant are internally used in Turkish folk medicine. Its use against diabetes is one of the prominent ones [5, 6]. *P. spina-christi* is antibacterial and antioxidant [7, 8]. In a study, *P. spina-christi* fruits extract decreased blood sugar and glycated hemoglobin levels on diabetic rats induced with streptozotocin [9]. The plant contains different classes of phytochemicals such as terpenes, flavonoids, tannins, amino acids, alkaloids and sterols [6, 7] which may cause the effects of plants to emergence. Thus, the phytochemical ingredient may be of vital importance.

One of the aims of our study is to isolate the secondary metabolites of *Paliurus spina-christi* and to elucidate their structures. Secondly, we aimed to investigate the antidiabetic potential of various extracts of fruits of *P. spina-christi* and its compounds isolated by us through enzyme inhibitory effect studies. The antidiabetic properties were evaluated based on *α*-glucosidase and *α*-amylase inhibitory studies. Various extracts (70% methanol, *n*-hexane, chloroform, ethyl acetate, *n*-butanol, water) and isolated compounds were tested. Our

aim was to obtain evidence of the traditional use of *P. spina-christi* as an antidiabetic agent and to evaluate the antidiabetic properties of its phytochemical components in terms of enzyme inhibitory effects.

Results And Discussion

Diabetes mellitus may be autoimmmune-origined or idiopathic (Type I DM) as well as caused by insulin resistance or relative insulin deficiency (Type II DM). Patients with type II DM comprises large majority of the people (90–95%), while the rest are suffering from type I DM, treatment of which only depends on insulin uptake. Vital organs such as eyes, kidneys, hearts exposing to oxidative stress and long-lasting hyperglycemia are under the risk for diabetes complications [10]. For this reason, antioxidant defense mechanisms as well as reducing hyperglycemia are of great importance.

Nowadays, medicinal plants offer drug candidate molecules for the treatment of diabetes as well as many diseases. Worldwide, more than 800 herbs have been reported for the treatment of diabetes [11]. Following the ethnobotanical research of natural products, it makes sense to conduct biological activity studies to provide evidence for their known activities. Plant phytocomponents 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 alpha glucosidase inhibitors rather than agents that inhibit both alpha amylase and alpha glucosidase could be of great importance to discover more reliable and less toxic drug candidates for type II DM. The antihyperglycemic effects of *Paliurus spina christi* fruit extracts prepared in different concentrations with solvents of different polarities and pure compounds isolated from this plant were evaluated as an indicator of their antidiabetic potential. 70% methanolic extract and its subfractions are polarity range of extraction solvents from non polar to polar respectively *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 were extracted.

Particularly, 70% methanolic (IC_{50} = 529.2 ± 38.6 µg/mL) and *n*-hexane (IC_{50} = 445.7 ± 8.5 µg/mL) extracts selectively and considerably inhibiting *α*-glucosidase may have significant potential and they showed higher activity than acarbose (IC_{50} = 4212.6 ± 130.0 µg/mL) (Table 1). Isolation studies were carried out on this extract since the highest effect was detected in *n*-hexane.

Three triterpenes were isolated. Betulin (**1a**) and betulinic acid (**1b**) were in a mixture. In addition lupeol (**2**) as the third terpenic substance and a sterol *β*-sitosterol (**3**) (Fig. 1), total of 4 compounds were isolated. The structure of the isolated compounds were identified by spectral methods, such as 1D (^{1}H-NMR, ^{13}C-NMR and DEPT), 2D (COSY, HMQC and HMBC), HRESIMS and by comparing with the literature. Betulin (**1a**), betulinic acid (**1b**) and lupeol (**2**) were isolated for the first time from this species.

Under the current research, lupeol, betulin, betulinic acid and *β*-sitosterol significantly inhibited *α*-glucosidase. Acarbose was used as positive control (IC_{50} = 6561 ± 207 µM).

Betulin (**1a**) and betulinic acid (**1b**) mixture showed highest activity (IC_{50} = 248 ± 12 µM). Further the other compounds lupeol (**2**) (IC_{50} = 2585 ± 79 µM) and *β*-sitosterol (**3**) (IC_{50} = 2939 ± 76 µM) were found also more active than acarbose (Table 4).

The strong *α*-glucosidase inhibitory activity of pentacyclic triterpenes may be attributed to the presence of functional groups (COOH and CH_2OH) at the C-28 position of the molecule [12]. Additionally all extracts and compounds were evaluated for their *α*-amylase inhibitory activities. None of them was found to be effective against the enzyme when compared with the acarbose as the positive control (IC_{50} = (IC_{50} = 1930 ± 46 µM).

Conclusion

In this study, we examined antidiabetic properties of *Paliurus spina-christi* fruit extracts and the phytoconstituents it contains, to form a basis for its ethnobotanical use in folk medicine. This research is the first evaluation on isolation of the potent compounds responsible for *α*-glucosidase inhibitory effect of *Paliurus spina-christi* fruits. In conclusion, extracts and phytocompounds with remarkable *α*-glucosidase inhibition potentials might be evaluated as significant resources, confirming the traditional use of *P. spina-christi* against diabetes. The *n*-hexane extract of *P. spina-christi* enriched in betulin and betulinic acid can be considered as new therapeutic candidates in the treatment of type II DM.

Experimental

General experimental procedures

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

Plant material

Paliurus spina-christi Mill. fruits were collected in Erzurum City, Uzundere province, Turkey, in July 2016. The plant material was authenticated by Forest Engineer, MSc Mehmet ÖNAL at the Eastern Anatolia Forestry Research Institute. A voucher specimen (No. AUEF 1348) was deposited at the Biodiversity Application and Research Center, Atatürk University, Erzurum, Turkey.

Paliurus spina-christi were dried under shade at room temperature until completely dried. After drying and removing the soil, the fruits are separated from plants and pulverized using a mechanical blender. The dried samples were then extracted with solvents of different polarity.

Extraction and isolation of pure compounds

Paliurus spina-christi Mill. dry fruits (800 g) were powdered and left to maceration overnight with 2 liters of 70% methanol and then extracted 3 times for 3 hours at 40°C using a mantle heater and reflux cooler. The filtered extracts were concentrated to dryness in the rotary evaporator at 40°C and 120 rpm. 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), respectively. 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 extracts were obtained. The isolation studies went on *n*-hexane extract, due to it showed best *α*-glucosidase inhibitory effect.

The *n*-hexane extract (2.18 g) was fractionated by a silica 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)$^+$ $C_{30}H_{50}O_2$; calcd. 442.3811) and 455.35860 (M-H)$^+$ $C_{30}H_{48}O_3$; calcd. 456.3603)], ^{1}H NMR and ^{13}C NMR data were identical to those reported in the literatures (Table 2) [13, 14, S1-S7].

Lupeol (Lup-20(29)-en-3*β*-ol) **(2)**: It was isolated as a white amorphous powder and its molecular formula, $C_{30}H_{50}O$, was determined by positive HRESIMS ion observed at *m/z* 409.38285 (M-OH)$^+$ (calcd. 426.3862). ^{1}H NMR and ^{13}C NMR data agree with the literature (Table 2) [15, S8-S13].

β-Sitosterol [(24R)-24Etilkolest-5-en-3β-ol] **(3)**: It was isolated as a white amorphous powder and its molecular formula, $C_{29}H_{50}O$, was determined by positive HRESIMS ion observed at *m/z* 414 (M$^+$) (calcd. 414.384). ^{1}H NMR and ^{13}C NMR data agree with the literature (Table 3) [16, S14-S19].

α-Glucosidase enzym inhibitory assay

The *α*-glucosidase inhibitory effect was determined as previously described method [17] with slight modifications as described before [18]. All 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. Mixture was incubated at 37°C for 5 min. Then *p*-nitrophenyl-*α*-D-glucopyranoside as substrate (20 µL, 3mM) was added for initiation of reaction and mixture was incubated at 37°C for 30 min. After incubation, 0.1 M sodium carbonate (50 µL) was added to all wells to quench the reaction. All solutions were prepared in a buffer system. Acarbose was used as a positive control. Amount of released *p*-nitrophenol was measured using a 96-well microplate reader at 405 nm. Each assay for all samples was performed in triplicate. Results are given as percentage inhibition

of enzyme activity and IC_{50} value. Percentage inhibition of all samples was calculated using the equation at below:

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

α-Amylase enzym inhibitory assay

The *α*-amylase inhibitory effect was determined as previously described method [19] with slight modifications as described before [18]. All 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 at 25°C for 10 min in 24-well microplate. 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, dinitrosalicylic 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. It was taken 50 µL from each well and then was added to 96-well microplate. The reaction mixture was diluted after adding 200 µL distilled water and absorbance was measured at 540 nm. Acarbose was used as a positive control. Each assay for all samples was performed in triplicate. Results are given as percentage inhibition of enzyme activity and IC_{50} value. Percentage inhibition of all samples was calculated using the equation at below:

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

Statistical analysis

All experiments were performed in triplicate. Kruskal-Wallis test was used to determine statistical significance. Results were analyzed using SPSS (IBM SPSS Statistics 20, IBM Corporation, Armonk, NY, USA) at the significance level of P = 0.05. The percent inhibition and IC_{50} value data for extracts and compounds are presented as means ± standard deviation.

Declarations

Conflict of Interest

The authors declare that they have no conflict of interest.

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Tables

Table 1
*α*Glucosidase inhibitory activities of extracts

α-Glucosidase inhibitory activity	IC_{50} 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 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[a]	4212.6	130.0	4222.8	4078.2	4337.7			

[a] Positive control for *α*–glucosidase inhibitory activity

Table 2

^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) datas of Betulin (**1a**), Betulinic acid (**1b**) and Lupeol (**2**) in DMSO-d_6 (δ in ppm, *J* in Hz)

	Betulin (1a)		**Betulinic acid (1b)**		**Lupeol (2)**	
Atom	**δ_C (ppm)**	**δ_H (ppm) J (Hz)**	**δ_C (ppm)**	**δ_H (ppm) J (Hz)**	**δ_C (ppm)**	**δ_H (ppm) J (Hz)**
1	38.75	0.86 (*m*) 1.63 (*m*)	38.75	0.86 (*m*) 1.63 (*m*)	38.69	1.64 (*m*) 0.85 (*m*)
2	26.89	1.54 (*m*)	26.89	1.54 (*m*)	27.14	1.57 (*m*) 1.65 (*m*)
3	78.69	3.13 (*dd*, *J* = 7.4/8.84)	78.69	3.13 (*dd*, *J* = 7.4/8.84)	78.88	3.14 (*dd*, *J* = 10.66/5.48)
4	37.1		37.10		37.12	
5	55.31	0.64 (*m*)	55.37	0.64 (*m*)	55.28	0.65 (*m*)
6	18.24	1.51 (*m*) 1.37 (*m*)	18.24	1.51 (*m*) 1.37 (*m*)	18.27	1.37 (*m*) 1.51 (*m*)
7	34.18	1.35/- (*m*)	34.30	1.35/-	34.25	1.23 (*m*) 1.36 (*m*)
8	40.85		40.64		40.80	
9	50.53	1.05 (*m*)	50.37	1.22 (*m*)	50.42	1.23 (*m*)
10	38.79	signal overlap	signal overlap		38.79	
11	20.78	1.41 (*m*) 1.23 (*m*)	20.85	1.41 (*m*) 1.23 (*m*)	20.90	1.23 (*m*) 1.40 (*m*)
12	25.20	1.63 (*m*) 1.04 (*m*)	25.50	1.63 (*m*) 1.04 (*m*)	25.12	1.07 (*m*) 1.63 (*m*)
13	37.25	1.66 (*m*)	38.25	1.66 (*m*)	38.03	1.65 (*m*)
14	47.63		42.62		42.79	
15	30.55	1.92 (*m*) -	29.10	1.92 (*m*) -	27.41	1.33 (*m*) 1.56 (*m*)

	Betulin (1a)		Betulinic acid (1b)		Lupeol (2)	
16	33.87	signal overlap	32.23	2.24/- (*m*)	35.55	1.29 (*m*) 1.49 (*m*)
17	42.39		56.19		42.96	
18	49.17	1.32 (*m*)	48.74	1.54 (*m*)	48.28	1.34 (*m*)
19	46.95	2.97 (*m*)	47.76	2.34 (*m*)	47.95	2.35 (*m*)
20	150.56		150.68		150.97	
21	29.66	1.90 (*m*) 1.22 (*m*)	29.62	1.90 (*m*) 1.22 (*m*)	29.80	1.23 (*m*) 1.89 (*m*)
22	38.75	signal overlap	38.75	1.39 (*m*)	39.96	1.38 (*m*) 1.19 (*m*)
23	27.78	0.91 (*s*)	27.78	0.91 (*s*)	27.90	0.93 (*s*)
24	15.79	0.78 (*s*)	15.26	0.72 (*s*)	15.32	0.73 (*s*)
25	15.98	0.79 (*s*)	15.95	0.79 (*s*)	16.05	0.80 (*s*)
26	15.79	1.00 (*s*)	15.79	1.00 (*s*)	15.92	1.01 (*s*)
27	14.60	0.94 (*s*)	14.55	0.94 (*s*)	14.49	0.92 (*s*)
28	59.73	3.25 (*d*, *J* = 10.92) 3.72 (*d*, *J* = 10.92)	179.14		17.94	0.76 (*s*)
29	109.35	4.52 (*bs)* 4.63 (*d*, *J* = 1.88)	109.42	4.54 (*bs)* 4.67 (*d*, *J* = 1.80)	109.25	4.53 (*dd*, *J* = 2.28/1.12) 4.65 (d, *J* = 2.20
30	18.93	1.64 (*s*)	19.16	1.65 (*s*)	19.24	1.65 (*s*)

Table 3

^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) data of *β*-Sitosterol (**3**) in DMSO-d_6 (δ in ppm, *J* in Hz)

Atom	δ_C (ppm)	δ_H (ppm), *J* (Hz)
1	37.26	
2	29.70	
3	71.83	3.54 (*m*)
4	42.33	2.29 (*m*)
5	140.76	
6	121.72	5.37 (*m*)
7	31.66	
8	31.92	
9	50.15	
10	36.51	
11	21.09	
12	39.78	
13	42.33	
14	56.78	
15	24.31	
16	28.25	
17	56.06	
18	11.86	0.70 (*s*)
19	19.40	1.02 (*s*)
20	36.15	
21	18.78	0.94 (d, *J* = 6.5)
22	33.96	
23	26.1	
24	45.85	
25	29.16	
26	19.82	0.84 (d, *J* = 7.04)

Atom	δ_C (ppm)	δ_H (ppm), *J* (Hz)
27	19.03	0.82 (d, *J* = 7.04)
28	23.08	
29	11.98	0.86 (*t*, *J* = 7.56)

Table 4
*α*Glucosidase inhibitory activities of isolated compounds

α-Glucosidase inhibitory activity	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			
β-Sitosterol (**3**)	2939	76	2911	2857	2965			
Acarbose[a]	6561	207	6577	6315	6723			

[a] Positive control for *α*–glucosidase inhibitory activity

Figures

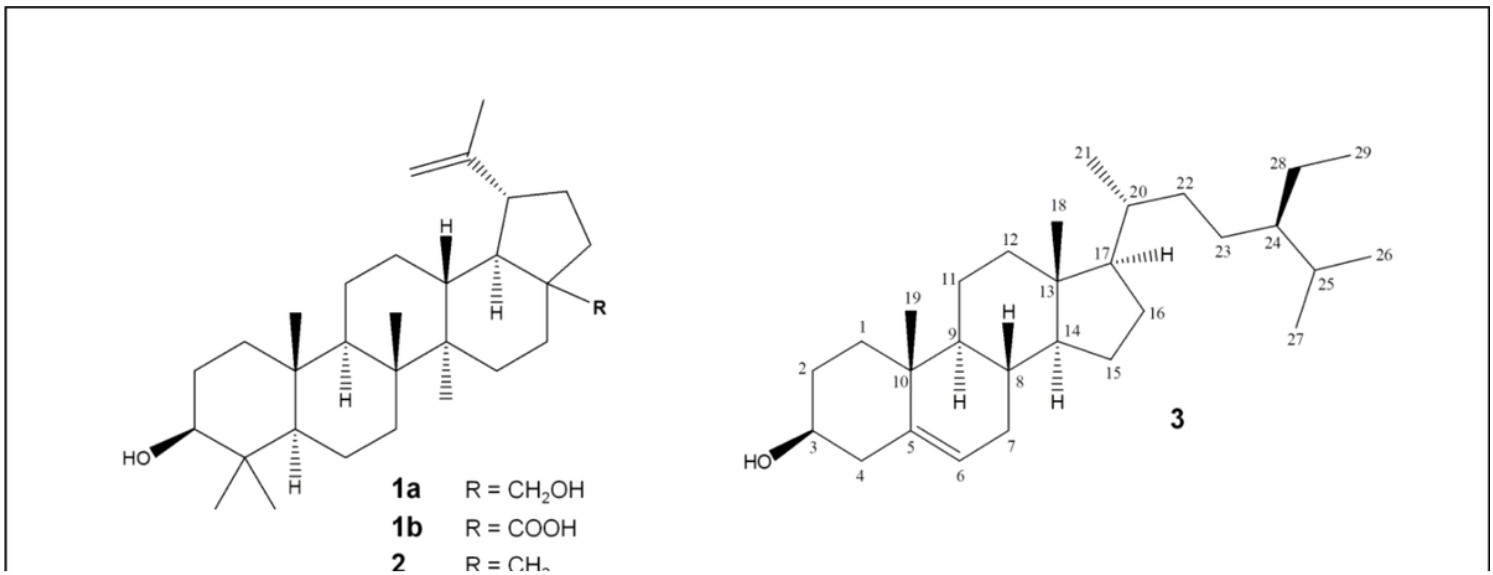

Figure 1

Chemical structures of compounds **1–3**

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