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α-Glucosidase and α-amylase inhibitory activities of saponins from traditional Chinese medicines in the treatment of diabetes mellitus

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Extracts of eleven traditional Chinese medicines (TCM) with a reputation of usefulness in treating diabetes mellitus were examined for α-glucosidase and α-amylase inhibitory activities *in vitro*. The extract with the highest activity was selected for further characterization. The extract of the root bark of *Aralia taibaiensis* (EAT) outperformed other extracts in the assays with IC_{50} values of 0.48 ± 0.01 mg/mL (BSG), 0.41 ± 0.02 mg/mL (SCG), 0.61 ± 0.03 mg/mL (BLA) and 0.77 ± 0.03 mg/mL (PPA), respectively. To identify which constituents were responsible for the activities, thirteen triterpenoid saponins were isolated from EAT and examined for their inhibitory effects against α-glucosidase and α-amylase. The results revealed that saponins **2**, **3**, **4** (IC_{50}: 0.83 ± 0.05 μM for BSG and IC_{50}: 0.72 ± 0.03 μM for SCG), **5**, **6**, **7**, **9**, **10**, **11** and **12** (IC_{50}: 1.07 ± 0.04 μM for BSG and IC_{50}: 0.93 ± 0.05 μM for SCG) showed α-glucosidase inhibitory activities, while **2**, **3**, **4** (IC_{50}: 0.93 ± 0.04 μM for PPA), **5**, **6**, **7**, **9**, **10**, **11** and **12** (IC_{50}: 1.02 ± 0.03 μM for PPA) possessed significant α-amylase inhibitory activities. In addition, the structure-activity relationship of the thirteen saponins was discussed based on their structure features and diabetes mellitus related activities. It is suggested that the glucuronic acid unit at C-3 of the aglycone is the imperative functional group of the antidiabetic activities, and two characteristic structural features are responsible for the remarkable α-glucosidase and α-amylase inhibitory activities.

1. Introduction

One therapeutic approach adopted to treating diabetes mellitus is to decrease postprandial hyperglycaemia by retarding absorption of glucose with inhibition of carbohydrate-hydrolysing enzymes, such as α-glucosidase and α-amylase, in the digestive tract (Bhandari et al. 2008; Shobana et al. 2009). Current examples of α-glucosidase and α-amylase inhibitors which are in clinical use, such as acarbose, miglitol, and voglibose, function directly in reducing the sharp increase in glucose levels that occurs immediately after food uptake (Saito et al. 1998; Jain and Saraf 2008). However, these non-specific enzyme inhibitors are known to produce serious side effects which include liver disorders, flatulence, abdominal pain, renal tumours, hepatic injury, acute hepatitis, abdominal fullness and diarrhoea (Fujisawa et al. 2005; Hiroyuki et al. 2001; Kim et al. 2004). Additionally, the current high prevalence of diabetes mellitus and prominent side effects of some oral hypoglycemic agents have led to an increasing need for seeking alternative therapies that may have less severe or no side effects.

Many medicinal plants have been reported to exhibit antidiabetic activity when assessed with presently available experimental models. Traditional Chinese medicines (TCM) continue playing an important role and show a bright future in the therapy of diabetes mellitus and related complications, particularly in some developing countries where most people have limited resources and no access to modern treatment (Jia et al. 2003). Indeed, many compounds isolated from TCMs show antidiabetic activity (Xi et al. 2008), of which saponins are promising compounds with the potential to be developed into new drugs against diabetes (Xu et al. 1996). Many studies have recently demonstrated that triterpenoid saponins are potently decreasing plasma glucose and plasma triglyceride levels (Oishi et al. 2007). A wide range of chemical classes indicate that a variety of mechanisms of action are likely to be involved in lowering blood glucose levels (Ali et al. 2006).

Despite that the antidiabetic TCM extracts rich in saponins play a major role in treating diabetes mellitus already, little information is available on their pharmacological properties. Therefore, eleven Chinese antidiabetic plants were selected on the basis of their traditional use, and their α-glucosidase and α-amylase activities were experimentally determined *in vitro*. In this study the extract of the root bark of *Aralia taibaiensis* (EAT) outperformed other extracts by its α-glucosidase and α-amylase inhibitory activities. To identify the constituents offering inhibitory activities, thirteen triterpenoid saponins were isolated from EAT and investigated for their α-glucosidase and α-amylase inhibition properties.

Table 1: Total saponin contents of eleven antidiabetic TCM extracts

Sample	total saponins content (OAE μg/mg extract)
PN	340.5 ± 3.98
PQ	411.9 ± 4.27
PC	104.1 ± 15.89
PH	321.2 ± 3.26
AA	332.1 ± 4.05
PT	221.7 ± 3.10
OJ	222.9 ± 4.52
AO	160.4 ± 2.06
AT	453.7 ± 2.62
AS	420.1 ± 12.30
AM	183.5 ± 6.38

The values are expressed as mean ± SD (n = 5). The contents of total saponins were expressed as oleanolic acid equivalents (OAE μg/mg extract).

2. Investigations and results

2.1. Total saponin content

The total saponin content determination showed that EAT possessed more saponins (45.37%, w/w) than the other extracts (Table 1). It should be noted that the vanillin-glacial acetic acid reagent gives only an approximate estimate of the total saponin content of TCM extracts.

2.2. α-Glucosidase and α-amylase inhibitory activities of the extracts

α-Glucosidase and α-amylase are key enzymes in the digestive system and catalyze the initial step in the hydrolysis of starch, a principal source of glucose in the diet (Sarangowa et al. 2010). Therefore, three kinds of α-glucosidase from different origins were used in our study: yeast α-glucosidase from *Bacillus stearothermophilus* (BSG), *Saccharomyces cerevisiae* (SCG) and mammalian α-glucosidase from rice (RG). Similarly, the following three kinds of α-amylase were used: bacterial α-amylase from *Bacillus licheniformis* (BLA), mammalian α-amylase from porcine pancreas (PPA) and human α-amylase from human saliva (HSA). This study was designed to establish the inhibitory activities of eleven extracts of TCM plants against α-glucosidase and α-amylase, digestive enzymes related to diabetes mellitus. The α-glucosidase and α-amylase inhibitory activities were compared with those of a commercial inhibitor, acarbose. The inhibitory effects on α-glucosidase and α-amylase of the extracts and acarbose are shown in Fig. 1 and Fig. 2. As observed, many extracts potently inhibited α-glucosidase and α-amylase, compared with acarbose, except for a few TCMs. EAT exerted the highest inhibitory activity against α-glucosidase and α-amylase enzymes in all of the eleven extracts, and its α-glucosidase and α-amylase inhibitory value is 67.86 ± 0.62% (BSG), 70.41 ± 0.82% (SCG), 35.07 ± 0.78% (RG), 55.97 ± 0.23% (PPA), 43.98 ± 0.31% (BLA) and 30.95 ± 0.56% (HSA) respectively, at the concentration of 1 mg/mL. The IC_{50} values of EAT against RG were higher than it against BSG, but the IC_{50} values of EAT against SCG were lower than it against BSG. And the IC_{50} values of EAT against HSA were higher than it against PPA, but the IC_{50} values of EAT against BLA were lower than it against PPA (Table 2). This trend was the same in the case of the extracts. This study has demonstrated that the inhibitory activity of EAT could be explained, at least in part, by its combined α-glucosidase and α-amylase inhibition properties.

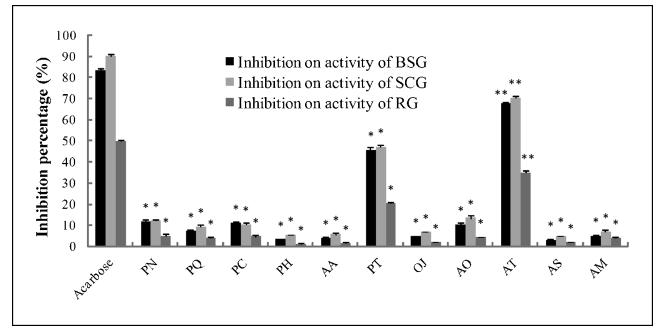

Fig. 1: α-Glucosidase inhibitory activities of eleven extracts of TCMs. The extracts were tested at a concentration of 1 mg/mL for inhibition of α-glucosidase. The values are expressed as mean ± SD (n = 5). $*P < 0.05$, $**P < 0.01$ compared with the control group

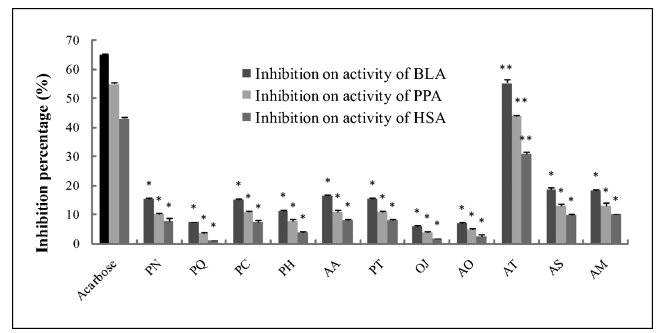

Fig. 2: α-Amylase inhibitory activities of eleven extracts of TCMs. The extracts were tested at a concentration of 1 mg/mL for inhibition of α-amylase. The values are expressed as mean ± SD (n = 5). $*P < 0.05$, $**P < 0.01$ compared with the control group

2.3. Activities of the isolated triterpenoid saponins

As EAT showed obviously higher inhibitory activities than the other extracts against α-glucosidase and α-amylase. To identify the main active compositions in EAT, the extract was further investigated by isolating the active constituents and evaluating the activities of the isolated triterpenoid saponins against α-glucosidase and α-amylase. In this study, EAT and thirteen pure triterpenoid saponins derived from EAT were tested for their effects on inhibition of α-glucosidase and α-amylase. As shown in Fig. 3 and Fig. 4, ten pure triterpenoid saponins displayed obvious inhibitory activities against α-glucosidase, and compound **12** was the most active agent in all of the tested compounds. Furthermore, ten saponins exhibited significant inhibitory activities against α-amylase. Both **4** and **12** possessed higher α-amylase inhibition activities than other triterpenoid saponins. Only for **4** and **12** inhibitory activities were determed (for IC_{50} values a Table 2). Although active saponins showed weaker inhibitory activities than the positive control acarbose (IC_{50}: 0.38 ± 0.03 μM for BSG, 0.32 ± 0.04 μM for SCG, 1.55 ± 0.04 μM for RG, 0.90 ± 0.02 μM for BLA and 0.79 ± 0.02 μM for PPA), these data clearly indicated the potential of these saponins as inhibitors of α-glucosidase and α-amylase. From all these observations, it can be concluded that the TCM extracts with a high level of saponins such as EAT showed excellent α-glucosidase and α-amylase inhibitory activities *in vitro*. These inhibitory activities of EAT and the active saponins might play important pharmacological roles in the prevention of diabetes mellitus and its complications.

3. Discussion

Our previous studies showed that the twelve triterpenoid saponins **1**–**12** isolated from EAT displayed certain diabetes

Table 2: Inhibitory effects of the extracts of EAT and pure triterpenoid saponins against α-glucosidase and α-amylase from different origins

Sample	IC_{50}					
	α-Glucosidase			α-Amylase		
	BSG	SCG	RG	BLA	PPA	HSA
Acarbose (mg/mL)	0.25 ± 0.02	0.21 ± 0.04	1.00 ± 0.03	0.90 ± 0.02	0.79 ± 0.02	> 1.00
Acarbose(μM)	0.38 ± 0.03	0.32 ± 0.04	1.55 ± 0.04	1.39 ± 0.02	1.22 ± 0.03	> 1.55
EAT (mg/mL)	0.48 ± 0.01**	0.41 ± 0.02**	> 1.00	0.61 ± 0.03**	0.77 ± 0.03**	> 1.00
4(μM)	0.83 ± 0.05*	0.72 ± 0.03*	> 1.00	> 1.00	0.93 ± 0.04*	> 1.00
12(μM)	1.07 ± 0.04*	0.93 ± 0.05*	> 1.28	> 1.28	1.02 ± 0.03*	> 1.28

*$P < 0.05$, **$P < 0.01$ compared with the control group

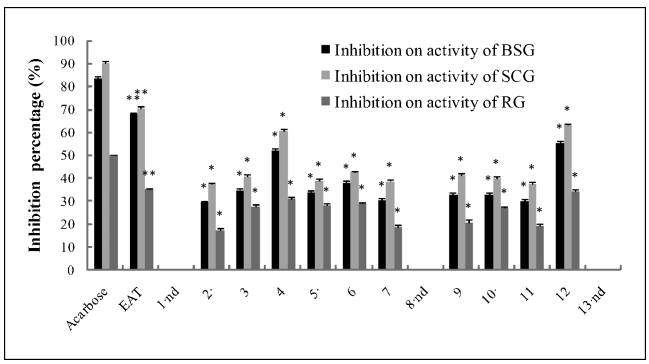

Fig. 3: α-Glucosidase inhibitory activities of EAT and thirteen pure triterpenoid saponins. The EAT and thirteen pure triterpenoid saponins were tested at a concentration of 1 mg/mL for inhibition of α-glucosidase. The values are expressed as mean ± SD ($n = 5$). *$P < 0.05$, **$P < 0.01$ compared with the control group

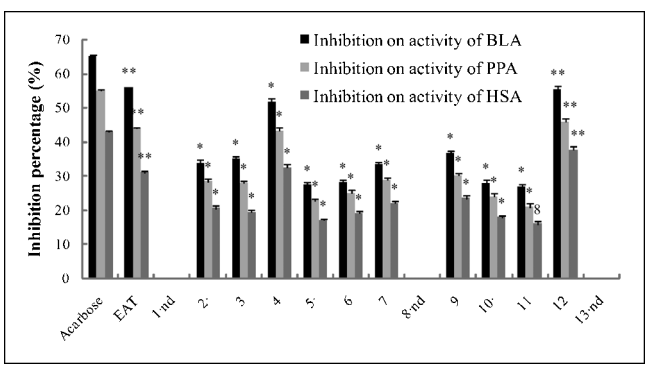

Fig. 4: α-Amylase inhibitory activities of EAT and thirteen pure triterpenoid saponins. The EAT and thirteen pure triterpenoid saponins were tested at a concentration of 1 mg/mL for inhibition of α-amylase. The values are expressed as mean ± SD ($n = 5$). *$P < 0.05$, **$P < 0.01$ compared with the control group

mellitus related antioxidant and antiglycation activities (Xi et al. 2010) and the primary structure-activity-relationships of several saponins have been discussed (Bi et al. 2012). We showed herein the α-glucosidase and α-amylase inhibition properties of EAT and the thirteen triterpenoid saponins **1**–**13** for the first time. In addition, further SAR of the thirteen saponins was discussed based on their structure features and diabetes mellitus related activities. Firstly, it seems that the glucuronic acid (GluA) unit at C-3 of the aglycone in these saponins is the imperative functional group of the antidiabetic activities by comparison of saponins **2**–**13** with **1**. Secondly, two characteristic structural features, i.e., the β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucuronopyranosyl oligosaccharide moiety or the β-D-glucuronopyranosyl moiety at C-3 of the aglycone in these saponins, seem to be responsible for the remarkable α-glucosidase and α-amylase inhibitory activities. When two glucose units were attached to C-2 and C-3 of GluA respectively (the first structural feature), the saponin showed higher activities, while the saponin was poorly active when the glucose unit was replaced by another sugar moiety, such as in saponins **2** and **7**. Saponin **4** was more active than **9**, indicating that the glucose moiety connected at C-28 of aglycone played an important role in terms of antidiabetes related activities. For the second structural feature, with a monosaccharide (GluA) at C-3 and a glucose moiety at C-28 of the aglycone, saponin **12** exhibited significant activity. In contrast, when an additional sugar moiety was attached to C-4 of GluA, saponin **8** showed no activity. However, saponin **11** shares the same sugar chain with **8** without a glucose moiety at C-28 of the aglycone. Saponin **11** exhibited moderate α-amylase inhibition activity. This suggested that the inhibitory activities are influenced by both the saccharide moiety at C-3 and the glucose unit at C-28 of the aglycone, and more samples should be tested to illustrate their definite SAR.

Interestingly, the partial results of antidiabetes related α-glucosidase and α-amylase inhibitory activities were consistent with our previously studied antioxidant and antiglycation activities, i.e., saponins **4**, **9** and **11** possessed the best antiglycation activities, and saponins **3**, **11** and **12** exhibited the best antioxidant activities (Xi et al. 2010). It seemed that saponins **4** and **12** showed stronger α-glucosidase and α-amylase inhibitory activities than **3**, **9** and **11**. Also the sensitiveness to different factors of the related antidiabetes activities was not completely coincident with each other. For example, saponin **3** possesed significant antioxidant and antiglycation activities (Bi et al. 2012) but showed weak α-glucosidase and α-amylase inhibitory activity. Therefore, we postulate that the different pharmacological effects and SAR of these saponins are caused by their different action targets and mechanisms. Furthermore, EAT was more active than any of its constituents (pure saponins) in all the assays. This might be ascribed to synergistic interactions among these saponins. Studies are continuing to explore and analyze the synergistic effect of the active triterpenoid saponins. The present studies provided strong evidence for further development of EAT and its active constituents in the treatment of diabetes mellitus.

4. Experimental

4.1. Plant materials

The eleven TCMs used in this study included root of *Panax notoginseng* Burk. F. H. Chen [Number (No.), SAPII001], root of *Panacis quinquefolii* (No. SAPII002), sclerotium of *Poria cocos* Schw. Wolf (No. SAPII003), root of *Pseudostellaria heterophylla* (No. SAPII004), rhizome of *Anemarrhena asphodeloides* (No. SAPII005), root bark of *Polygala tenuifolia* Willd (No. SAPII006), root of *Ophiopogon japocicus* Thunb. Ker-Gawl (No. SAPII007), root of *Asparrausi officinalis L.* (No. SAPII008), root bark of

Aralia taibaiensis Z. Z. Wang et H. C. Zheng (No. SAPII009), root of *Acanthopanax senticosus* Rupr. et Maxim. Harms (No. SAPII010) and root of *Astragalus membranaceus* Bunge (No. SAPII011). All TCMs were bought from a local market in Xi'an in May 2009. The plants were botanically identified by Dr. Haifeng Tang, professor of the Department of Pharmacy, Xijing Hospital, Fourth Military Medical University. The voucher specimens were deposited in the Herbarium of Department of Pharmacy.

4.2. Extraction of plant materials

Before the experiment, eleven dried TCMs were ground into powder via a blender. The appropriate dried powdered plant material (20 g) was successively extracted three times with 80% (v/v) ethanol (herb:ethanol = 1:10, w/v) under reflux (80 °C) for 60 min, and filtered. The supernatant was concentrated via rotary evaporation, suspended in distilled water and then partitioned successively with chloroform (ratio 1:3, v/v) and *n*-butanol saturated with water (ratio 1:3, v/v, three times). The *n*-butanol extracts were taken to dryness, under reduced pressure at 60 °C using a rotary vacuum evaporator. The eluent was collected on the evaporating dish and evaporated at 60 °C in a water bath.

The percentage yields (w/w) obtained for the various plant extracts were as follows: *Panax notoginseng* (1.05%, PN), *Panacis quinquefolii* (1.06%, PQ), *Poria cocos* (2.41%, PC), *Pseudostellaria heterophylla* (0.92%, PH), *Anemarrhena asphodeloides* (1.50%, AA), *Polygala tenuifolia* (3.73%, PT), *Ophiopogon japocicus* (1.18%, OJ), *Asparrausi officinalis* (5.67%, AO), *Aralia taibaiensis* (0.82%, AT), *Acanthopanax senticosus* (1.21%, AS) and *Astragalus membranaceus* (3.65%, AM). Most of these extracts were easily soluble in 20 mM phosphate buffer (pH 6.9). The extracts of PT and PC needed to be ultrasounded. A fresh solution of extract was prepared for each assay.

4.3. Determination of total saponin content

The total saponins content of each extracts was determined approximately using the method described by Wang Y with a slight modification (Wang et al. 2006). For 5% vanillin-glacial acetic acid solution, 0.25 g vanillin was transferred to a 5 mL volumetric flask, and then the solution was shaken until the vanillin was totally dissolved. To 10 mL tubes fitted with plugs, 0.2 mL reference standard solution, 0.2 mL sample solution and 0.2 mL methanol were added. Then, the solvent in each tube was evaporated at 60 °C in a water-bath. The residue was dissolved in 0. 2 mL 5% vanillin and mixed with 0.8 mL perchloric acid, placed in a water bath at 60 °C for 15 min, quickly cooled by ice water, and then 5 mL acetic acid was added to each tube. Lastly, absorbance was measured at 553 nm by spectrophotometer. Oleanolic acid was used as a reference standard and the content of total saponins was expressed as oleanolic acid equivalents (OAE, μg/mg extract)

4.4. Isolation and identification of triterpenoid saponins from EAT

The detailed isolation procedure of thirteen pure triterpenoid saponins from the crude extract (total saponins) of *Aralia taibaiensis* has been described previously (Tang et al. 1996, 1997) and their structures were identified by comparison of their spectral data (IR, ^{1}H NMR, ^{13}C NMR, ESI-MS) and chemical evidence (GC/MS analysis of the acid hydrolysates) with literature values (Fig. 5). All these triterpenoid saponins were easily soluble in 20 mM phosphate buffer (pH 6.9). A fresh solution of the triterpenoid saponins was prepared for each assay.

4.5. Assessment of α-glucosidase inhibitory activity

α-Glucosidase inhibition assay was performed as described previously with a slight modification (Kim et al. 2004). The reaction mixture comprised: 20 μL of 0.2 M potassium phosphate buffer (pH 6.8), 20 μL of 1 mg/mL test sample, 50 μL of 1 mg/mL reduced glutathione, 20 μL of 1 U/mL α-glucosidase. After pre-incubation at 37 °C for 10 min, 50 μL of 5 mM pNPG was added. The enzymatic reaction was allowed to proceed at 37 °C for 15 min and was stopped by the addition of 1000 mL of 0.2 M Na_2CO_3. p-Nitrophenol absorption was measured using a microplate reader (Tecan Group Ltd., Austria) at 400 nm. Solution without sample was used as a control. Solution without substrate and sample was used as a blank. Acarbose (1 mg/mL) was also assayed as a standard reference. The experiment was performed in quintuple. The inhibition percentage of α-glucosidase was calculated as Inhibition (%) = $\{1 - (A_{400nm}$ sample $- A_{400nm}$ blank$)/A_{400nm}$ control$\} \times 100$, where A_{400nm} sample represents the absorbance of the experimental sample, A_{400nm} blank represents the absorbance of the blank, and A_{400nm} control represents the absorbance of the control. This study was designed to establish the inhibitory activity of eleven extracts of TCM plants and 13 pure triterpenoid saponins isolated from EAT against α-glucosidase and α-amylase. The activities of extracts and triterpenoid saponins were assessed by plotting percentage inhibition against a range of concentrations and by determining the IC_{50} value.

4.6. Assessment of α-amylase inhibitory activity

Pancreatic α-amylase inhibitory activity was determined by the chromogenic method described by Sigma–Aldrich with a slight modification (Ali et al. 2006). A total of 10 μL of sample (1 mg/mL in deionized water), 190 μL of deionized water, 200 μL of α-amylase inhibitor and 200 μL of porcine pancreatic α-amylase solution (1 unit/mL in cold deionized water) were incubated at 25 °C for 5 min. After preincubation, 200 μL of 1% potato starch solution in 20 mM sodium phosphate buffer (pH 6.9 with 6.7 mM NaCl) was added to each tube. The reaction mixtures were then incubated at 25 °C for 3 min. The 100 μL reaction mixtures were removed and added into a separate tube containing 50 μL DNS colour reagent solution (96 mM 3,5-dinitrosalicylic acid, 5.31 M sodium potassium tartrate in 2 M NaOH). The test tubes were then incubated in a boiling water bath for 15 min and cooled down to room temperature. The reaction mixtures were then diluted with 450 μL deionized water. α-Amylase inhibitory activity was measured using a microplate reader (Tecan Group Ltd., Austria) at 540 nm. Acarbose was used as the positive control. The absorbance (A) due to maltose generated was calculated as: A_{540nm} control or sample = A_{540nm} test $- A_{540nm}$ blank. The α-amylase inhibitory activity was calculated according to the equation below: Inhibition (%) = $(1 - A_{540nm}$ sample$/A_{540nm}$ control$) \times 100$.

4.7. Statistical analysis

Statistical analysis was performed with an analysis of variance (ANOVA) followed by LSD test for multiple comparisons. Data were analyzed by SPSS v.18.0 software package and expressed as mean ± SD. Differences were considered significant at $P < 0.05$.

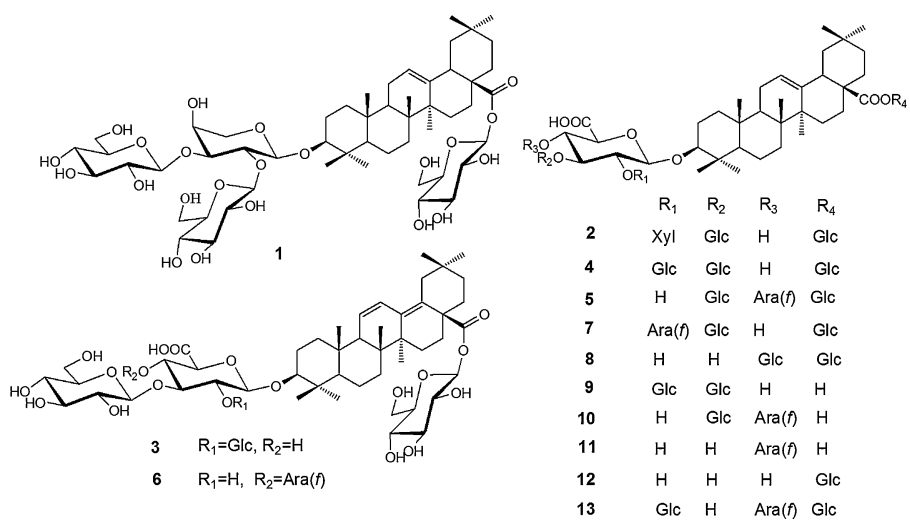

Fig. 5: Structures of thirteen triterpenoid saponins isolated from the extract of root bark of *Aralia taibaiensis*. Ara(*f*): α-L-arabinopyranosyl, Glc: β-D-glucopyranosyl, Xyl: β-D-xylopyranosyl

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