Modulation of Kv7 Channel Currents by Echinocystic Acid

DanDan Geng¹#, Yaning Li¹#, Rong Zheng¹, Runmeng Wang¹, Bo Yang¹, Huaxing Zhang², Yang Zhang³, Fan Zhang¹*

¹The Key Laboratory of Neural and Vascular Biology, Ministry of Education; Department of Biochemistry and Molecular Biology, Hebei Medical University, Shijiazhuang, China

²The Core Facilities and Centers, Hebei Medical University, Shijiazhuang, China

³School of Pharmacy, Hebei Medical University, Shijiazhuang, China;

#These authors contributed equally

*Correspondence to:

Fan Zhang, Ph.D.

Department of Biochemistry and Molecular Biology, Hebei Medical University, Shijiazhuang, China. 050017

zhangfan86@hebmu.edu.cn

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**Abbreviations:** HEK293: human embryonic kidney 293; IC$_{50}$: half maximal inhibitory concentration; DRG: Dorsal root ganglion
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Abstract

Modulation of KCNQ-encoded voltage-gated potassium Kv7/M channel function represents an attractive strategy to treat neuronal excitability disorders such as epilepsy, pain, and depression. The Kv7 channel group includes five subfamily members (Kv7.1–7.5). Pentacyclic triterpenes display extensive pharmacological activities including anti-tumor, anti-inflammatory, and anti-depression effects. In this study, we investigated the effects of pentacyclic triterpenes on Kv7 channels. Our results show that echinocystic acid, ursonic acid, oleanonic acid, demethylzeylasteral, corosolic acid, betulinaldehyde, acetylursolic acid, and α-boswellic acid gradually exert decreasing degrees of Kv7.2/Kv7.3 channel current inhibition. Echinocystic acid was the most potent inhibitor, with a half-maximal inhibitory concentration (IC₅₀) of 2.5 µM. It significantly shifted the voltage-dependent activation curve in a positive direction and slowed the time constant of activation for Kv7.2/Kv7.3 channel currents. Furthermore, echinocystic acid non-selectively inhibited Kv7.1–Kv7.5 channels. Taken together, our findings indicate that echinocystic acid is a novel and potent inhibitor that could be used as a tool to further understand the pharmacological functions of neuronal Kv7 channels.
Significance Statement

Pentacyclic triterpenes reportedly have multiple potential therapeutic uses such as anticancer, anti-inflammatory, antioxidant, and anti-depression effects. In the present study, we show that echinocystic acid, ursonic acid, oleanonic acid, and demethylzeylasteral inhibit Kv7.2/Kv7.3 channels to varying degrees. Of these, echinocystic acid was the most potent Kv7.2/Kv7.3 current inhibitor and inhibited Kv7.1–Kv7.5 currents in a non-selective manner.
Introduction

KCNQ-encoded voltage-gated potassium Kv7/KCNQ/M channels generate a negative activation threshold (approximately -60 mV), slow activation and deactivation kinetics K+ currents, and do not inactivate to suppress neuronal excitability, which is involved in neuropsychiatric disorders such as epilepsy (Jentsch, 2000; Peters et al., 2005; Rogawski and Bazil, 2008; Shah et al., 2013), pain (Wang et al., 2021; Wickenden and McNaughton-Smith, 2009), depression (Peng et al., 2017), schizophrenia (Peng et al., 2017), Parkinson’s disease (Shi et al., 2013), and Alzheimer’s disease (Peng et al., 2017). Kv7 potassium channel subtypes are composed of five members (Kv7.1–Kv7.5). The Kv7.1 channel is predominantly expressed in cardiac smooth muscle cells (Brown and Passmore, 2009; Stott et al., 2014), and loss-of-function mutations in Kv7.1 lead to long QT syndrome (Park et al., 2005; Wang et al., 1996). The remaining four isoforms (Kv7.2–Kv7.5) are mainly expressed in the central and peripheral nervous systems (Robbins, 2001). Co-assembly of Kv7.2 and Kv7.3 channels is believed to be the major component of native M currents, which play a critical role in controlling neuronal membrane excitability (Wang et al., 1998). Accordingly, modulators of the Kv7/M channel have become a focus as tools to understand mechanisms of channel biophysical properties and develop clinical therapeutic agents.

Natural products play an essential role in new drug research and development processes (Petrovska, 2012). Pentacyclic triterpenes are primary and secondary plant
metabolites of pomaces, flowers, fruit peels, stems, leaves, and barks of various plants; indeed, triterpenes comprise up to 30% of the dry weight of a few species (Jäger et al., 2009). It has been estimated that more than 20,000 triterpenoids are available in nature (Sheng and Sun, 2011). Pentacyclic triterpenes such as echinocystic acid (Yu et al., 2019), ursonic acid (Sun et al., 2020), oleanonic acid (Castellano et al., 2022), demethylzeylasteral (Yang et al., 2020), corosolic acid (Qian et al., 2021), betulinaldehyde (Chung et al., 2022), acetylursolic acid (Wafaa et al., 2015), and ⍺-boswellic acid (Ammon, 2006) have multiple biological activities. Echinocystic acid extract from the fruits of *Gleditsia sinensis* Lam., displays a range of biological activities including anti-cancer, anti-inflammatory, anti-oxidant, anti-depression, anti-colitis, and anti-diabetic effects (Deng et al., 2015; Kumar et al., 2012; Li et al., 2016; Yang et al., 2016). Ursonic acid can be isolated from *Ziziphus jujuba* Mill., a traditional medicine (Song et al., 2020), and is well-known for its wide range of beneficial effects, such as anti-cancer, anti-inflammatory, anti-oxidant, anti-diabetic, anti-bacterial, anti/protozoal, and anti-viral activities (da Silva et al., 2019; Son and Lee, 2020a, b; Woźniak et al., 2015; Yin et al., 2018; Zhang et al., 2017). Oleanonic acid, present in a limited number of natural resources, has been shown to suppress the activity of human immunodeficiency virus 1 (Pollier and Goossens, 2012). Demethylzeylasteral, an extract of *Tripterygium wilfordii* Hook F, also displays multiple biological activities such as anti-cancer, anti-inflammatory, immunosuppression, and anti-atherosclerosis effects (An et al., 2015; Ma et al., 2007; Wang et al., 2017; Zhang et al., 2018).
In the present study, we show that echinocystic acid, ursonic acid, oleanonic acid, demethylzeylasteral, corosolic acid, betulinaldehyde, acetylursolic acid, and α-boswellic acid exert varying degrees of inhibition on Kv7.2/Kv7.3 channel currents. Among them, echinocystic acid was the most potent inhibitor, with an IC₅₀ of 2.5 µM that is similar to XE991.

Materials and Methods

Compounds

Echinocystic acid, ursonic acid, oleanonic acid, demethylzeylasteral, corosolic acid, betulinaldehyde, acetylursolic acid, and α-boswellic acid were purchased from TOPSCIENCS (ShangHai, China). Each drug was dissolved in saline (0.9%) and diluted to the desired concentration before each experiment and kept away from light exposure. XE991 [10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone] was purchased from Sigma (Burlington, MA, USA).

DNA constructs

Plasmids in a pcDNA3.1 vector encoding human Kv7.1 (GenBank Accession Number NM000218), human Kv7.2 (AF110020), rat Kv7.3 (AF091247), human Kv7.4 (AF105202), and human Kv7.5 (AF249278) were generated and kindly provided by Diomedes E. Logothetis (Virginia Commonwealth University, Richmond, VA, USA) and used for transfection and expression in HEK293 cells (Zhang et al., 2019a).

Cell culture
Human embryonic kidney 293 (HEK293) cells were cultured at 37°C in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum and antibiotics. For transfection of four wells of cells, a mixture of 2 µg Kv7, 2 µg green fluorescent protein pcDNA, and 3 µL of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was prepared in 0.6 mL of DMEM and then applied to cells for 4–6 h. Electrophysiological recordings were made 24–48 h after transfection (Zhang et al., 2019a).

**Electrophysiological recordings**

Current recordings were performed using a perforated patch-clamp technique with amphotericin B (250 mg·mL⁻¹, Sigma) at room temperature. The signals were amplified using an HEKA EPC10 patch-clamp amplifier (HEKA Elektronik, Lambrecht, German). Current records were acquired at 10 kHz and filtered at 2.5 kHz. Pipettes were pulled from borosilicate glass capillaries with a micropipette puller (Sutter Instruments, Novato, CA, USA). Pipettes were filled with intracellular solution of the following composition: KCl 150, MgCl₂ 5, HEPES 10 at pH 7.4 adjusted with KOH, and had a final resistance of 1–2 MΩ. Series resistances were all compensated and set to 60%–80%. The external solution for recording of HEK293 cell lines was as follows (in mM): NaCl 160, KCl 2.5, MgCl₂ 1, CaCl₂ 2, glucose 10, HEPES 20 and pH 7.4 adjusted with NaOH (Zhang et al., 2019a).

**Computational simulation**

*Discovery Studio 2020 (DS-2020)* was used for the prediction of binding sites and molecular docking. The required Kv7.2 crystal was derived from Protein Data Bank.
Bank Entry ID 7CR1 (Li et al., 2021) and then processed by DS-2020. First, the Prepare Proteins tool in the Macromolecules module was applied to optimize the protein crystal, including removal of water and heteroatoms, addition of hydrogens, completion of the loop region, and protonation of the protein. Next, the From Receptor Cavities tool in the Receptor-Ligand Interactions module was used to predict hydrophobic pockets of the receptor. The molecular structure of echinocystic acid was sketched by ChemDraw 19.0, which was processed by Prepare Ligands in Small Molecules module. LibDock protocol was adopted to dock echinocystic acid in the predicted binding sites.

In addition, the GPU-accelerated PMEMD program in AMBER20 software was used to perform molecular dynamic (MD) simulation of the established research systems. The ff14SB force field and gaff force field in the LEaP module were used to produce corresponding topology (*.prmtop) and coordinate files (*.inpcrd). Five-thousand steps each of the steepest descent method and conjugate gradient method were applied to minimize the energy of constructed systems (Li et al., 2022; Zhang et al., 2020). Finally, a 100-ns MD simulation was carried out in the NPT ensemble and conformational and energetic analyses were conducted based on the resulting MD trajectory files.

**Data analysis and statistics**

Dose-response curves were fitted by the logistic equation: \( y = A_2 + \frac{(A_1 - A_2)}{(1 + (x/x_0)^p)} \), where \( A_2 \) is the minimum drug concentration, \( A_1 \) is the maximum drug concentration, \( x \) is the drug concentration, \( x_0 \) is the IC\(_{50}\), and \( p \) is the Hill coefficient.
Channel conductance (G) was calculated by using Ohm’s law: \( G = I / (V - V_{rev}) \), where \( I \) corresponds to the current amplitude at the end of the pulse, \( V \) is the membrane potential, and \( V_{rev} \) is the calculated reversal potential (-85.5 mV). The Boltzmann function was used to fit the conductance-voltage curves and to determine the maximal conductance \((G_{max})\) and half-maximal activation voltage of Kv7 currents, where \( k \) is the slope factor and \( G = G_{max}/ \{1 + \exp \left[-(V - V_{1/2})/k\right]\} \). Activation and deactivation currents were fitted to the single exponential function: \( I = A \times [1 - \exp(-t/\tau)] \), where \( I \) is the current, \( A \) is the amplitude, \( t \) is time, and \( \tau \) is the time constant. Origin9.1 software is used to perform these fits. Results are expressed as mean ± SEM.

Results

**Pentacyclic triterpenes inhibits Kv7.2/Kv7.3 channel currents**

We first studied the effect of pentacyclic triterpenes on exogenous Kv7.2/Kv7.3 channels expressed in HEK293 cells (Figure 1A, C, E, G, I, K, M, O). The results shown in Figure 1B, D, F, H, J, L, N, P are typical current traces (a slowly activating and deactivating, non-inactivating outward current) elicited by stepwise depolarization from the holding potential of -80 mV to 0 mV. 10 μM echinocystic acid produced a reversible inhibitory effect on Kv7.2/Kv7.3 currents by 93.8% ± 3.9% (n = 6); whereas, 30 μM ursonic acid, oleanonic acid, demethylzeylaetal, corosolic acid, betulinaldehyde, acetylursolic acid, and α-boswellic acid inhibited Kv7.2/Kv7.3 currents by 80.4% ± 4.4% (n = 6), 52.8% ± 2.3% (n = 6), 45.5% ± 3.5% (n = 6), 44.3% ± 2.5% (n = 6), 26.6% ± 2.1% (n = 6), 13.3% ± 1.1% (n = 6), and 12.1% ± 0.9% (n = 6), respectively (Figure 1Q).
Echinocystic acid, ursonic acid, oleanonic acid, and demethylzeylasteral inhibit Kv7.2/Kv7.3 currents in a concentration-dependent manner

Administration of different concentrations of echinocystic acid (Fig. 2A), ursonic acid, oleanonic acid, and demethylzeylasteral generated dose-dependent inhibition of Kv7.2/Kv7.3 channel currents. As shown in Fig. 2B, fitting of the dose-response curve yielded IC\textsubscript{50} and Hill coefficient values (respectively) of 2.5 ± 0.1 µM and 1.6 ± 0.1 (n = 6), 7.4 ± 0.4 µM and 1.4 ± 0.1 (n = 6), 11.2 ± 0.4 µM and 2.0 ± 0.1 (n = 6), and 9.4 ± 3.3 µM and 0.9 ± 0.2 (n = 6) for echinocystic acid, ursonic acid, oleanonic acid, and demethylzeylasteral, respectively.

We next evaluated the effect of echinocystic acid, ursonic acid, oleanonic acid, and demethylzeylasteral on voltage-dependent activation of Kv7.2/Kv7.3 currents. The results shown in Figure 3A are representative currents traces elicited by a train of voltage steps from -80 to +40 mV by increasing 10-mV intervals with a holding potential set at -80 mV. Echinocystic acid, ursonic acid, oleanonic acid, and demethylzeylasteral significantly inhibited maximal activation of Kv7.2/Kv7.3 currents. Echinocystic acid (10 µM), ursonic acid (30 µM), oleanonic acid (30 µM), and demethylzeylasteral (30 µM) shifted the voltage for V\textsubscript{1/2} of Kv7.2/Kv7.3 channels toward more depolarized potentials, from -29.2 ± 0.5 to 18.6 ± 3.0 mV (n = 8), from -30.5 ± 1.3 to 2.2 ± 1.2 mV (n = 6), from -28.1 ± 1.7 to 0.3 ± 2.0 mV (n = 6), from -28.6 ± 0.7 to 3.4 ± 0.9 mV (n = 6), respectively (Fig. 3B). Echinocystic acid, ursonic acid, oleanonic acid, and demethylzeylasteral produced a substantial rightward shift of
the V$_{1/2}$ of Kv7.2/Kv7.3 currents by 47.8 mV (n = 8), 32.7 mV (n = 6), 28.4 mV (n = 6), and 32 mV (n = 6), respectively. Echinocystic acid, ursonic acid, oleanonic acid, and demethylzeylasteral reduced the maximum conductance of Kv7.2/Kv7.3 channels by 90.1% ± 3.1% (n = 8), 72.1% ± 2.7% (n = 6), 49.5% ± 1.8% (n = 6), 63.1% ± 2.1% (n = 6), respectively. As summarized in the results shown in Figures 1–3, echinocystic acid was the most potent Kv7.2/Kv7.3 channel inhibitor.

**Effect of echinocystic acid on Kv7.1-Kv7.5 channel currents**

To explore the selectivity of echinocystic acid on Kv7 subfamily members, typical homo- and heterotetrameric Kv7.1–Kv7.5 channel currents elicited by voltage steps from -80 to +40 mV. In particular, Kv7.5 channel expressed alone did not produce significant currents, therefore it was co-expressed with Kv7.3 and the effect of echinocystic acid was tested on heterologous Kv7.3/Kv7.5 currents. As shown in Figure 4, echinocystic acid completely inhibited Kv7.1–Kv7.5 channel currents. Indeed, Kv7.1, Kv7.2, Kv7.3, Kv7.4, and Kv7.3/Kv7.5 channel currents were inhibited by 98% ± 2.7% (n = 6), 96% ± 3.0% (n = 7), 100% (n = 6), 100% (n = 6), and 97.2% ± 2.1% (n = 6), respectively, following treatment with 10 µM echinocystic acid at +40 mV.

**Preliminary prediction of the binding sites of echinocystic acid on Kv7.2**

To predict binding sites, three reliable sites with promising docking scores (docking score: **Site 3**, 122.65; **Site 6**, 111.64; **Site 10**, 96.08) were selected for subsequent
molecular (MD) dynamic simulation (Fig. 5A). To comprehensively evaluate binding interactions between echinocystic acid and these sites, 100-ns MD simulations were conducted on the initial binding conformations built by LibDock, and root mean square deviation (RMSD) representative conformation and energy analysis were performed on the simulated trajectories. As shown in Fig. 5B, all the research systems could reach dynamic equilibrium with the extension of simulation time. Compare with Site 3 (RMSD \(\approx 3.56\) Å), residues at Site 6 (RMSD \(\approx 1.27\) Å) and 10 (RMSD \(\approx 1.34\) Å) were less volatile, which was more favorable for binding of echinocystic acid. Additionally, representative conformations of the studied systems were extracted from dynamic equilibrium trajectories and superimposed onto their corresponding initial conformations. As shown in Fig. 5C, Sites 3, 6, and 10 were mainly composed of rigid \(\alpha\)-helices and during the dynamic simulation, ligands deflected in the binding pockets to varying degrees. Echinocystic acid had the least deflection on Site 10, indicating that it was relatively stable at this site. Moreover, decomposition free energy calculation was carried out to identify the key amino acids contributing to ligand binding at each site. Key residues with absolute energy contributions \((|\text{energy contribution}|) \geq 0.5\) kcal/mol were summarized. There were 24 and 25 key residues on Site 6 and Site 10 that were conducive to the binding of echinocystic acid. Particularly, Q321 on Site 10 could form a relatively stable hydrogen bond with echinocystic acid, and the hydrogen bond formation accounting for 32.26% of binding during the whole MD simulation process, further stabilizing ligand binding (Fig. 5D).

**Discussion**
Pentacyclic triterpenes are medicinally important agents with pharmacologically potent constituents (Sharma et al., 2018; Sheng and Sun, 2011). Numerous studies have demonstrated their effectiveness for the treatment of cancer (Paduch and Kandefer-Szerszen, 2014; Park et al., 2021), diabetes (Alqahtani et al., 2013; Oboh et al., 2021), obesity (Rao et al., 2011), inflammation (Safayhi and Sailer, 1997; Wang et al., 2017), and other conditions (Abdullah et al., 2022; Patocka, 2003). In this study, we demonstrate that eight pentacyclic triterpenes inhibited Kv7.2/Kv7.3 channel currents in HEK293 cells. Our results also show that echinocystic acid, ursonic acid, oleanonic acid, and demethylzeylasteral inhibited Kv7.2/Kv7.3 currents in a dose-dependent manner and shifted the V_{1/2} of Kv7.2/Kv7.3 currents toward more depolarized potentials.

In recent years, multiple compounds capable of inhibiting Kv7/M channel currents have been reported. Linopirdine is a classical inhibitor that exhibits potent inhibition of both recombinant and native Kv7/M currents (Aiken et al., 1995; Schnee and Brown, 1998). Subsequently, XE991, DMP 543, UCL2077, ML252 and HN38 were developed to exhibit higher potency (Cheung et al., 2012; Earl et al., 1998; Hu et al., 2013; Soh and Tzingounis, 2010). By screening a natural product library, we identified several pentacyclic triterpenes that inhibited Kv7.2/Kv7.3 currents and found that the inhibitory effects of echinocystic acid, ursonic acid, oleanonic acid, demethylzeylasteral, corosolic acid, betulinaldehyde, acetylursolic acid, and α-boswellic acid gradually decreased. The most potent inhibitor was echinocystic acid (97% inhibition rate) while the least potent inhibitor was α-boswellic acid (12.1%
inhibition rate). Furthermore, IC₅₀ values for inhibition of Kv7.2/Kv7.3 currents by echinocystic acid, ursonic acid, oleanonic acid, and demethylzeylasteral were 2.5 ± 0.1 µM, 7.4 ± 0.4 µM, 11.2 ± 0.4 µM, and 9.4 ± 3.3 µM, respectively.

Thus, we focus on echinocystic acid, which was the most potent inhibitor. Several outstanding features of echinocystic acid can be summarized based on its effects on Kv7 currents. First, the inhibitory effect of echinocystic acid is potent; specifically, the potency of echinocystic acid in inhibiting the Kv7.2/Kv7.3 channel is similar to that of XE991 (1 µM) (Greene et al., 2017; Wang et al., 1998). However, the effect of echinocystic acid on Kv7 channels was reversible, while the effect of XE991 was irreversible (Brueggemann and Byron, 2012). Second, echinocystic acid caused a remarkable rightward shift of voltage-dependent inhibition of Kv7.2/Kv7.3 channels toward more positive potentials. Echinocystic acid also significantly slowed Kv7.2/Kv7.3 channel activation and accelerated deactivation kinetics. Third, application of 10 µM echinocystic acid resulted in 98%, 96%, 100%, 100%, and 97% inhibition of Kv7.1, Kv7.2, Kv7.3, Kv7.4, and Kv7.3/Kv7.5 channels, respectively. Thus, echinocystic acid inhibited Kv7.1–Kv7.5 channels in a non-selective manner. Collectively, these characteristics of echinocystic acid indicate that it may be a useful tool to increase understanding of the pharmacological and biological physical functions of neuronal Kv7 channels.

Kv7/M channel inhibitors can be developed to improve defective neuronal activity, such as that caused by Alzheimer’s and Parkinson's diseases (Bian et al., 2020; Liu et al., 2018; Miceli et al., 2018; Peng et al., 2017). Unfortunately, both
linopirdine and XE991 did not pass Phase 3 clinical trials. However, XE991 has been widely used in cell culture and animal experiments to investigate the physiological and pathological processes of Kv7/M channels (Bian et al., 2020; Greene et al., 2017; Liu et al., 2018). Interestingly, echinocystic acid was found to enhance spatial learning and memory in aged mice by promoting neuron outgrowth through the c-Jun N-terminal kinase signaling pathway, and ameliorated scopolamine-induced memory and learning deficits in mice by inhibiting acetylcholinesterase activity and inducing expression of brain-derived neurotrophic factor and phosphorylate-cAMP response element-binding protein (Jung et al., 2012; Park et al., 2017). In addition, echinocystic acid may improve movement disorders associated with Parkinson’s disease by reducing MPTP-induced damage of dopaminergic neurons by inhibiting neuroinflammation (He et al., 2021). Thus, our findings suggest that the inhibitory effect of echinocystic acid on Kv7/M channels may enhance depolarization-induced transmitter release underlying a novel mechanism for the treatment of Alzheimer’s and Parkinson’s diseases.

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Authorship Contributions

Participated in research design: F Zhang.
Conducted experiments: Geng, Zheng and Y Zhang.

Contributed new reagents or analytic tools: H.X. Zhang.

Performed data analysis: Wang, Yang and Li.

Wrote or contributed to the writing of the manuscript: F Zhang.

References


through the Intrinsic Apoptosis and Stat3 Signaling Pathways in Osteosarcoma.

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Figure Legends

**Figure 1** The effects of pentacyclic triterpenes on Kv7.2/Kv7.3 channel currents in HEK293 cells. Structures of echinocystic acid (A), ursonic acid (C), oleanolic acid (E), demethylzeystasteral (G), corosolic acid (I), betulinaldehyde (K), acetylursolic acid (M), and α-boswellic acid (O) are shown. Typical outward currents elicited by stepwise depolarization from a holding potential of −80 mV to 0 mV in the absence or presence of 10 µM echinocystic acid (B, n = 6), 30 µM ursonic acid (D, n = 6), 30 µM
oleanonic acid (F, n = 6), 30 µM demethylzeylasteral (H, n = 6), 30 µM corosolic acid (J, n = 6), 30 µM betulinaldehyde (L, n = 6), 30 µM acetylursolic acid (N, n = 6), and 30 µM α-boswellic acid (P, n = 6) are shown. (Q) Histogram plotting the summary of effects of pentacyclic triterpenes on Kv7.2/Kv7.3 channel currents (n = 6).

**Figure 2** Echinocystic acid, ursonic acid, oleanonic acid, and demethylzeylasteral inhibit Kv7.2/Kv7.3 channel currents in a concentration-dependent manner. (A) Typical outward currents elicited by stepwise depolarization from a holding potential of −80 mV to 0 mV in the absence or presence of 0.3, 1, 3, 10, or 30 µM echinocystic acid. (B) Logistic fitting of concentration-dependent inhibition of Kv7.2/Kv7.3 currents by echinocystic acid, ursonic acid, oleanonic acid, and demethylzeylasteral revealed half-maximal inhibitory concentration (IC50) values of 2.5 ± 0.1 µM (n = 6), 7.4 ± 0.4 µM (n = 6), 11.2 ± 0.4 µM (n = 6), and 9.4 ± 3.3 µM (n = 6), respectively.

**Figure 3** Effect of echinocystic acid, ursonic acid, oleanonic acid, and demethylzeylasteral on the voltage dependence of Kv7.2/Kv7.3 channel activation. A series of outward currents was elicited by depolarizing voltage steps (holding at −80 mV, with voltage steps increasing in 10-mV increments from −80 to +40 mV) in the absence and presence of 10 µM echinocystic acid (A), 30 µM ursonic acid (C), 30 µM oleanonic acid (E), and 30 µM demethylzeylasteral (G). Summary of the relative G-V relationship of Kv7.2/Kv7.3 channels without and with echinocystic acid (B, n = 8), ursonic acid (D, n = 6), oleanonic acid (F, n = 6), and demethylzeylasteral (H, n = 6) and fitted with the Boltzmann function.
Figure 4 Echinocystic acid inhibits Kv7.1–Kv7.5 channel currents. Kv7.1 (A, n = 6), Kv7.2 (B, n = 7), Kv7.3 (C, n = 6), Kv7.4 (D, n = 6), and Kv7.3/Kv7.5 (E, n = 6) channel currents were elicited by depolarizing voltage steps (holding at −80 mV, increasing in 10-mV increments from −80 to +40 mV) in the absence and presence of echinocystic acid (10 μM). (F) Histogram plotting the summary of effects of pentacyclic triterpenes on Kv7.1–Kv7.5 channel currents.

Figure 5 (A) Predicted binding sites of echinocystic acid on Kv7.2. (B) Root-mean-square deviations of binding site residue backbone atoms and ligand heavy atoms as a function of time in MD simulations. (C) Structural superimposition of the initial docking conformations and representative conformations. (D) Per-residue binding free energy decomposition of key residues with energy contribution (≥ 0.1 kcal/mol).
Figure 2
Figure 3

A

500 pA

250 ms

Control

Echinoceystic acid

B

Relative conductance (G)

Voltage (mV)

C

500 pA

250 ms

Control

Ursolic Acid

D

Relative conductance (G)

Voltage (mV)

E

500 pA

250 ms

Control

Oleanonic Acid

F

Relative conductance (G)

Voltage (mV)

G

100 pA

250 ms

Control

Demethyrlaxasterol

H

Relative conductance (G)

Voltage (mV)
Figure 4

Control and Echinocystic acid (10 μM) effects on Kv7.1-7.5 channels.

A. Kv7.1
B. Kv7.2
C. Kv7.3
D. Kv7.4
E. Kv7.3/7.5

F. Summary graph showing % inhibition for each channel.

% Inhibition:
- Kv7.1: 98%
- Kv7.2: 97%
- Kv7.3: 100%
- Kv7.4: 100%
- Kv7.3/7.5: 96%
Figure 5