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Original Research Article

The Effects of Hydro-Alcoholic Extracts of *Glycyrrhiza inflata* for Review on Voltage-Gated Sodium Channel Subtype 1.4

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Abstract : Licorice is an anti-spasmodic drug for treating gastrocnemius spasm, and it is well-known that licorice targets voltage-gated sodium channels (VGSCs). Herein, researchers studied the effect of *G. inflata* on Nav1.4 Na^+ currents using CHO cells expressing human Nav1.4 VGSCs. On treating *G. inflata*, it is showed that 4 mg/ml hydroalcoholic extracts of *G. inflata* at 30%, 50%, 70% and 90% (v/v) acquired 100.00%, 96.70%, 32.14% and 48.18% inhibition on $I_{Nav}1.4$. By contrast, at 8 mg/ml we found that at 30%, 50%, 70% and 90% (v/v), inhibition rates of 100.00%, 10.0%, 100.00% and 100.00% on $I_{Nav}1.4$ were respectively seen. Treatment with echinatin, isoliquiritigenin, glycyrrhizic acid and liquiritin exhibited higher inhibition rates of 33.20%, 39.98%, 20.54% and 22.62% respectively. And the concentrations ranged from 0.30μg/ml, 0.17μg/ml, 16.74μg/ml and 43.70μg/ml to 1.12μg/ml, 0.32μg/ml, 39.47μg/ml, 122.58μg/ml. Nevertheless, neoisoliquiritin, glycyrrhetinic acid, formononetin, and liquiritigenin exhibited lower inhibition rates about 20%. Furthermore, treatment with isoliquiritin, liquiritin apioside and neoliquiritin had negligible effects on $I_{Nav}1.4$. It is found that glycyrrhizic acid attained a maximum concentration of 122.58μg/ml, but echinatin achieved the lowest concentration. The hydroalcoholic extracts of *G. inflata* had obvious inhibitory effects on Nav1.4 VGSCs which may represent a very important mechanism in treating gastrocnemius. Meanwhile, it can also guide further study regarding the material basis and therapeutic mechanism for gastrocnemius spasm with a peony and licorice decoction and other Traditional Chinese Medicines containing glycyrrhiza.

Keywords: Gastrocnemius spasm; *Glycyrrhiza inflata* (*G. inflata*); patch-clamp; skeletal muscle; Nav1.4 Voltage-Gated Sodium Channels (VGSCs).

1. INTRODUCTION

Voltage-gated sodium channels (VGSCs) widely exist in the neurons, muscles and various excitable cells of the central and peripheral nervous systems [1], and play a significant role in the excitation of various neurons. As a type of transmembrane protein, sodium channel proteins introduce extracellular sodium ions into cell membranes to produce action potentials (APs) during local depolarization of the cell membrane. Therefore, these proteins take a critical role in the generation and transmission of APs [2].

VGSCs are extremely important in the propagation and generation of APs in the excitable tissues, for example the muscle, nerve and heart [3]. As is now well-established, VGSCs are essential for the resting potential, they are also crucial in the generation and propagation of APs in neurons [4]. Thus far, researchers have found at least nine distinct sodium channel isoforms in the nervous system. The most recent research indicates that VGSCs take a significant role in normal neuronal electrophysiological activities and also they are closely correlated with the development of nervous system diseases [5]. Research has previously shown that nervous system VGSCs are closely associated with neurological diseases including neuropathic pain, brain tumors and multiple sclerosis [6].

Extensive research suggests that VGSCs are important; they are composed of an α-subunit and a **β**-subunit. To date, researchers have found nine α-

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subunits (i.e., Nav1.1~1.9) and four **β**-subunits (**β**1 - **β**4) in mammalian systems [7, 8]. It is noteworthy that Nav1.4 is primarily expressed in skeletal muscles [9]. It is also quite well-established that five common skeletal muscle diseases that include potassium-aggravated myotonia (PAM), hyperkalemic periodicparalysis (HyperPP), paramyotonia congenita (PMC), congenital myasthenic syndrome (CMS) and a form of hypokalemic periodic paralysis (HypoPP) exhibit a strong correlation with Nav1.4 which is expressed fundamentally in the skeletal muscle [10-11]. Thus, this channel is considered a target when treating spasm, abnormal muscle contractility and paralysis [12].

The gastrocnemius muscle is a typical skeletal muscle tissue which lies on top of the soleus that runs from the knee to the ankle joints. It is often used to study the motor system, due in part, to its important functional roles in the motor system [13]. So far, the main mechanism responsible for the development or appearance of gastrocnemius lesions remains unclear. However, many studies have shown that gastrocnemius lesions can give rise to many unintended bad consequences, including gastrocnemius spasm [14]. In addition, these types of painful spasms have a higher incidence [15]. Traditional Chinese medicine holds that common causes of leg spasm include cold stimulation, rapid muscle contraction, excessive sweating, excessive fatigue and calcium deficiency. One of the most authoritative explanations defined this suggests the mutation of Nav1.4 [16] leads to rapid inactivation of the sodium channel after it opens, and on doing so, adds a standing current flow to the inside, which leads to ceaseless muscle depolarization and undue muscle fiber stimulation that can finally lead to muscle spasm [17]. The relationship between Nav1.4 and the gastrocnemius spasm has been an ongoing focus of research.

In traditional Chinese medicine, licorice is one of the most common herbs. And it was considered as "top grade" in Shen Nong Ben Cao Jing which indicated that the herbs are nontoxic. There are three different original plants of licorice stipulated in the China Pharmacopeia. These include Glycyrrhiza uralensis Fisch., G. glabra L., and G. inflata Bat. However, formerly researches have shown that the three licorices exhibited quite different pharmacodynamic effects, especially in the treatment of muscular spasm. In the final analysis, the main reason for this observation might be associated with the differential content and chemical composition of the known licorices.

Licorice has been used for thousands of years in traditional Chinese medicine. And its prescriptions have been used to treat various diseases since ancient times in China, particularly in conditions that include gastrocnemius spasm, gastric spasm, and intestinal spasm, among many others [18, 19]. One of the most classic prescriptions, which are still in clinical use today, is peony and licorice decoction. Many clinical and experimental studies have confirmed that peony and licorice decoction can harmonize the physiological functions of the liver and spleen and relieve cramps and pain [20]. Further, peony and licorice decoction has a significant therapeutic efficacy on gastrocnemius spasm and it is also a classical prescriptions in treating leg cramps (modern medicine calls it gastrocnemius spasm) [21, 22].

We conjecture that the active chemical compounds in licorices play an anti-spasmodic effect by restraining Nav1.4 VGSCs based on the conclusions mentioned above. In our previous work, we explored the effects of a 4 and 8 mg/ml hydroalcoholic extracts (30%, 50%, 70% and 90%, v/v) of G. uralensis on human Nav1.4 VGSCs, which were steadily expressed in CHO cells. We found that this extract exhibited an acceptable inhibitory effect on $I_{Nav}1.4$ [23]. Additionally, we tested four marker chemical compounds of G. uralensis in the sake of studying the mechanism and material basis of treating gastrocnemius by using the UPLC-DAD method.

Herein, we studied the inhibitory action of a 4 and 8 mg/ml hydroalcoholic extracts (30%, 50%, 70% and 90%, v/v) of G. inflata on human Nav1.4 VGSCs, which were steadily expressed in CHO cells. In addition, we studied activation and inactivation kinetics, the recovery curve, frequency and concentration-dependent inhibitory effects and determined the IC50 of the hydroalcoholic extracts of G. inflata at 50% (v/v) on Nav1.4. The over-arching aim was to enable a comparison of the inhibitory effects of different original plants of licorice on Nav1.4 VGSCs, and to research the therapeutic mechanism of action and material basis when treating gastrocnemius spasm with the classic prescription of peony and licorice decoction.

2. MATERIALS AND METHODS

2.1 Drugs, chemicals, reagents and instrumentations

Licorices were cultivated for three years and gathered from Aksu (Xinjiang Uygur Autonomous Region) and was identified as Glycyrrhiza inflata Bat by Dr. Zhang Peng.

Reference compounds were purchased from Chengdu Pufeide Biotech Co., Ltd. of China (Chengdu, China), including glycyrrhizic acid (No.150407, purity≥98%), isoliquiritin (No.150714, purity≥98%), liquiritin (No.151013, purity≥98%), liquiritigenin (No.150511, purity≥98%), echinatin (No.160417, purity≥98%), isoliquiritigenin (No.141020, purity≥98%),formononetin (No.160821, purity≥98%), glycyrrhetinic acid (No.150723, purity≥98%), neoisoliquiritin (No.150913, purit≥98%), liquiritin apioside (No.160408, purity≥98%) and neoliquiritin (No.150819, purity≥98%).

Data acquisition and analysis was done using an Agilent ultra-performance liquid chromatography system (Agilent 1290 Infinity II, USA). The LC system was equipped with a DAD detector (G7117A, USA), an Agilent 1290 infinity column heater (G7166B, USA), an Agilent 1290 auto-sampler (G7167B, USA) and a Thermo Accucore-C18 (1.5 × 210 mm, 2.6 μm) column (Thermo Fisher Scientific, USA).

The extracellular solution consists of 40 mM tetraethylammonium-Cl, 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES buffer and 5 mM D-Glucose monohydrate. In the end, the solution was tuned up to pH 7.4 with NaOH. The internal pipette solution consists of 0.1 mM $CaCl_2$, 145 mM $CsCl_2$, 2 mM $MgCl_2$, 0.5 mM Na2-GTP,10 mM NaCl, 1.1 mM EGTA, 2 mM Mg-ATP and 10 mM HEPES buffer, which was tuned up to pH 7.2 with CsOH.

In this study, we used a PCR instrument (2720, USA), a gene sequencer (3730, USA), a micropipette puller (P97, USA), a microscope (IX71, Japan), a capillary glass tube (BF150-86-10, USA), a microelectrode manipulator (MP285, USA), an electronic analytical balance (BP110S, Germany) and an amplifier (EPC10, Germany).

2.2 UPLC-DAD determination

2.2.1 Sample preparation

For LC analyses, the extracts solution was dissolved in methanol (v/v, 1:1). Before injection, the solution was centrifuged at 12000 rpm for 5 min. At last, it was filtered through a 0.22 μm microporous membrane filter.

2.2.2 Separation conditions

The gradient elution system consisted of formic acid water (0.1% v/v) (solvent A) and acetonitrile (solvent B). The optimal separation was described thus: 0_5 min, 5%_10% (B); 5_10 min, 10%_15% (B); 10_18 min, 15% _20% (B); 18_25 min, 20%_25% (B); 25_35 min, 25%_40% (B); 35_36 min, 40%_95% (B); 36_46 min, 95% (B). The column and the auto-sampler temperature were maintained at 30°C and 25°C respectively. The flow rate was set at 0.4 ml/min. The wavelengths were 270 nm for liquiritin, 230 nm for glycyrrhizic acid, and 360 nm for echinatin and isoliquiritigenin. The injection volume was set at 3 μl.

2.2.3 Preparation of standard solutions

Glycyrrhizic acid, liquiritin, echinatin and isoliquiritigenin were respectively weighed and then dissolved together with methanol to get a working solution of four various concentrations which were 88 μg/ml, 66 μg/ml, 76 μg/ml and 68 μg/ml. The standard curves were constructed by testing the mixed solution, and the series of working solutions were within the ranges of 1.76_24.64 μg/ml for glycyrrhizic acid, 1.32_18.48 μg/ml for liquiritin, 1.52_21.28 μg/ml for isoliquiritigenin and 1.36 19.04 μg/ml for echinatin. The solutions were all stored at 4°C and in dark brown volumetric flasks.

2.2.4 Method validation

The LC method was tested and verified in the light of linearity, precision, stability, repeatability and recovery. The data was showed as mean ± one standard deviation (STDEV) about the mean, and the Relative Standard Deviation (RSD) was used to estimate precision, repeatability, stability and recovery.

2.3 Cell culture

Recombinant SCN4A was expressed in CHO cells. The SCN4A cDNA was strictly similar to the GenBank accession number NM_000334.4. Cells expressing channels were cultured in F12 medium that was replenished with 0.8 mg/ml G418 and10% FBS in the culture flasks. Cells were grown under an atmosphere of 5% carbon dioxide in air in a fully humidified incubator at 37°C. For patch-clamp experiments, cells were seeded at a density of 3×10^3 in a 24-well plate (final medium volume: 500 μl) with one coverslip in each well and a test duration of 18 h.

2.4 Stable expression of human Nav1.4 in CHO cells

Nav1.4 cDNA was subcloned into the pCDNA3.1 expression vector (Thermo Fisher Scientific). This vector contains both a CMV and an SV40 promoter, which drives the expression of the inserted target cDNA and the geneticin-resistant gene, respectively. The CHO-K1 cells were transfected with this construct using Lipofectamine 2000 reagent (Thermo Fisher Scientific). After transfection, cells were permitted to grow and express the protein for antibiotic resistance under non-selective conditions.

Medium was removed 48 r post-transfection, and cells were rinsed once with PBS, following which, the adherent cells were detached using TrypL Express solution (Gibco). The cells were harvested using Ham's F12 culture medium (HyClone) that was supplemented with 10% FBS (Gibco) and 1.6 mg/ml antibiotic that was pretested for the host cell. Cells were seeded into a 96-well plate with at most two cells in each well. It is important to thoroughly suspend the cells before seeding, and to avoid harsh treatment by frequent pipetting. Cells were incubated under standard conditions and were feed after two weeks with fresh selection medium. The host cell was also prepared in parallel as a null control. Cell clones were tested as soon as the cells in the non-transfected control wells had completely died. Then, once the resistant clones were identified, cells were expanded in culture in 6-well plates and assayed for the expression of the gene of interest using a manual patch clamp test. Positive clones were expanded and cryopreserved in a timely manner, and a stable cell-line was routinely maintained at half concentration of the selection antibiotic.

2.5 Electrophysiology

2. 5.1 Sample preparation

Medicinal materials were immersed in hydroalcoholic at the concentration of 30%, 50%, 70% and 90% (v/v) respectively. All the medicinal materials were boiled twice for 60 min respectively. For electrophysiology research, each of the freeze-dried powder was dissolved in DMSO (1%, v/v) to obtain 4 and 8 mg/ml stock solutions (calculated with crude drugs).

2. 5.2 Electrophysiological recordings

The electrophysiological recordings were obtained under visual control of a microscope. To measure the Nav1.4 channels, the membrane potential was held at -90 mV, and then depolarized to -10 mV for 50 ms to activate Nav1.4 currents. An interpulse interval of 10 s allowed recovery from inactivation.

The data were stored and analyzed with Patch master and Igor Pro, and were expressed by $\bar{X} \pm S$. Sodium currents from activation were converted to sodium conductance [G = I/(V-Vrev)] and plotted as a function of the test potential using the Boltzmann equation [G/Gmax = 1/(1+exp((Vh-V)/K)] to give values for Vh (potential causing half-maximal activation) and K (the slope factor). Similarly, currents from steady-state inactivation were also plotted as a function of the prepulse potential and fitted with the Boltzmann equation [I/Imax = 1/(1+exp((V-Vh)/K)] to give values for Vh (i.e., the potential causing half-maximal activation) and K (the slope factor). Currents from the steady-state recovery process curves were plotted as a function of the prepulse potential and fitted with the Boltzmann equation [$I = A_1 \exp(-t/\tau) + A_0$] to give values for τ (recovery time).

3. RESULTS

3.1 DNA barcoding

The splicing sequence was compared with the database and the standard reference sequence of G. inflata after sequencing.

(1) ITS2 test sequence of G. inflate

CAGACCGTTGCCCGACAACAATTGCCTCGCGAT
AGGTACTTTGGTTGTGCAGGGTGAATGTTGGCT
TCCCGTGAGCATTGCGGCCTCACGGTTGGCTCA
AAACTGAGTCCATGGTAGGGTTTGGCATGATCG
ATGGTGGTTGAGTGACGCTCGAGACCAATCAT
GTGTGACTCCACTGAGTTTGGGCTCTGTAACCA
ATAGGCGTCTTTGAACGCTCGTGATG.

(2) PsbA-trnH test sequence of G. inflata.

CCATCTATAAATGGATAATATTTTGGTTTTAAA
GAAGGATACGAGGTTTTGAAAGTAAAGGAGTA
ATATCAACATTGTTGATATTACTCCCTTCTTGAC
TTTTACTTTTCTTAGTAGTCTATATATGTATATA
TATACATACATATTGTAATACATATGACTTCAC
AATGTAAAATCAGAAAAAAAGAAATGTTTTCT
TATTTTTTCTGATTTTCTCGTATTTTAGAAGACG
CGTAAGAACTTAAAAGAGAAGAAAATAAGTGA
TAATGAAAAAGTCTAAATGGAAAATTAGATAA
TTTATACAT.

3.2 Content of echinatin, isoliquiritigenin, glycyrrhizic acid and liquiritin in the hydroalcoholic extracts of G. inflata

3.2.1 Calibration curves

Linearity was tested by analyzing six injection quantities of standard solutions. In addition, the calibration curves were constructed by plotting the peak areas and the injection quantities of each chemical compound with six distinct concentrations. The linear regression equations for echiantin, liquiritin, glycyrrhizic acid and isoliquiritigenin were defined as follows: y = 4768324.747x + 5.726; y = 3697762.509x + 2.241; y = 1112094.785x - 1.900; y = 8823821.909x + 5.948. The derived correlation coefficients were: 0.998, 1.00, 1.00 and 1.00. The linear ranges were found to be 1.36 - 19.04 μg/ml; 1.32 - 18.48 μg/ml; 1.76 - 24.64 μg/ml and 1.52 - 21.28 μg/ml.

3.2.2 Precision, stability, repeatability and recovery

Six replicate determinations of the standard working solution were tested to validate the precision. The solutions were stored at about 25℃for up to 24 hours and which was analyzed at different time-points (0, 4, 8, 12 and 24 h after preparation) to test the stability of the solutions. The results showed that the four chemical compounds were stable within 24 h at about 25°C and the RSD values range from 1.99% to 1.35 %. Repeatability was tested by six replicates of one sample. Finally, in the recovery test, the samples were prepared at three different concentration levels in triplicate by spiking known quantities of each of the four standards into the G. inflata sample solution, after which the extraction and analysis were done in the light of the described procedures. The data of the validation are given in Table 1.

Table-1: Precision, stability, repeatability and recovery of the four standard substances

Standard Substance	Precision RSD (%) (n=6)	Stability RSD (%) (n=6)	Repeatability RSD (%) (n=6)	Recoveries (%) (n=9)	RSD (%)
liquiritin	1.45	1.09	1.67	97.77	1.28
echinatin	1.12	1.13	2.07	99.63	1.28
isoliquiritigenin	1.53	1.35	1.58	97.88	2.03
glycyrrhizic acid	1.67	1.28	2.46	99.59	2.11

(a) Recovery (%) = 100*(amount found-original amount)/amount spiked

3.2.3 Content

The data demonstrated that echiantin, liquiritin, glycyrrhizic acid and isoliquiritigenin were showed in the hydroalcoholic extracts of G. inflata (Figure 1). The contents of the four marker compounds in the hydroalcoholic extracts of G. inflata are shown in Table 2.

Table-2: Contents of the four compounds in the hydroalcoholic extracts of G. inflata

Groups	Concentration (mg/ml)	Liquiritin (μg/ml)	Echinatin (μg/ml)	Isoliquiritigenin (μg/ml)	Glycyrrhizic acid (μg/ml)
hydroalcoholic extracts (v/v, 30%) of G. inflata	4	16.74	0.17	0.30	43.70
	8	33.49	0.34	0.60	87.40
hydroalcoholic extracts (v/v, 50%) of G. inflata	4	15.75	0.28	0.34	44.44
	8	31.49	0.56	0.68	88.87
hydroalcoholic extracts (v/v, 70%) of G. inflata	4	10.21	0.36	0.40	75.18
	8	20.43	0.72	0.80	150.36
hydroalcoholic extracts (v/v, 90%) of G. inflata	4	19.74	0.16	0.56	61.29
	8	39.47	0.32	1.12	122.58

3.3 Inhibitory effect of the hydroalcoholic extracts of G. inflata on Nav1.4

3.3.1 Effect of the hydroalcoholic extracts (30%, 50%, 70% and 90%, v/v) of G. inflata on Nav1.4

In this research, the whole-cell patch clamp technique was used to study the effect of the hydroalcoholic extracts (30%, 50%, 70% and 90%, v/v) of G. inflata on the $I_{Nav}1.4$. The peak current significant decreased after the treatment with 4 and 8 mg/ml hydroalcoholic extracts of G. inflata at 30% (v/v) as compared with the control group ($P < 0.05$; $n = 3$). The $I_{Nav}1.4$ decreased from the value of -1872.97, -1662.20 and -2111.10 pA to 0.00, 0.00 and 0.00 pA with 4 and 8 mg/ml hydroalcoholic extracts respectively. Additionally, the inhibitory effect of the 4 mg/ml group was the same as that seen for the 8 mg/ml group (Figure 2A1). The inhibition rate of both groups on $I_{Nav}1.4$ was 100%. The inhibition effect of the hydroalcoholic extracts of G. inflata at 30% (v/v) on the $I_{Nav}1.4$, appeared within 4 min after initiating the perfusion of the bath solution which contains the hydroalcoholic extracts of G. inflata at 30% (v/v) (Figure 2A2).

The peak current significant decreased after the treatment with 4 and 8 mg/ml hydroalcoholic extracts of G. inflata at 50% (v/v) as compared with the control ($P< 0.05$; $n = 3$). The $I_{Nav}1.4$ decreased from -580.81, -1093.9 and -693.68 pA to -20.00, 0.00 and -44.74 pA respectively, and the inhibition rate on $I_{Nav}1.4$ was 96.55%, 100.0% and 93.55% respectively with 4 mg/ml hydroalcoholic extracts. The $I_{Nav}1.4$ decreased from -580.81, -1093.9 and -693.68 pA to -0.00, .00 and 0.00 pA respectively, and the inhibition rate on $I_{Nav}1.4$ was 100.00%, 100.0% and 100.00% respectively with 8 mg/ml hydroalcoholic extracts. There was no significant difference in the inhibition rate when comparing the 8 and the 4 mg/ml groups ($P < 0.05$; $n=3$; Figure 2B1). This inhibitory effect of the hydroalcoholic extracts of G. inflata at 50% (v/v) on the $I_{Nav}1.4$ appeared within 4 min on initiating perfusion of the bath solution which contains the hydroalcoholic extracts of G. inflata at 50% (v/v) (Figure 2B2).

The peak current significant decreased following the treatment with 4 and 8 mg/ml hydroalcoholic extracts of G. inflata at 70% (v/v) as compared with the control ($P< 0.05$; $n = 3$). The $I_{Nav}1.4$ decreased from -5578.00, -975.30 and -1087.80 pA to -3954.80, -587.43 and -787.97 pA respectively, and the inhibition rate on $I_{Nav}1.4$ was 29.10%, 39.77% and 27.56% respectively with 4 mg/ml hydroalcoholic extracts. The $I_{Nav}1.4$ decreased from -975.30, -1087.80 and -527.98 pA to -0.00, 0.00 and 0.00 pA respectively, and the inhibition rate on $I_{Nav}1.4$ was 100.00%, 100.0% and 100.00% respectively with 8 mg/ml hydroalcoholic extracts. The inhibition ratio of the 8 mg/ml group was markedly higher than the 4 mg/ml group ($P< 0.05$; $n = 3$) (Figure 2C1). This inhibition ratio of the hydroalcoholic extracts (v/v, 70%) of G. inflata at 70% (v/v) on the $I_{Nav}1.4$, appeared within 3 min after starting the perfusion of the bath solution which contains the hydroalcoholic extracts of G. inflata at 70% (v/v) (Figure 2C2).

The peak current significant decreased after the treatment with 4 and 8 mg/ml hydroalcoholic extracts of G. inflata at 90% (v/v) as compared with the control ($P< 0.05$; $n = 3$). The $I_{Nav}1.4$ decreased from -2244.60, -5140.60 and -1133.90 pA to -1285.70, -2595.80 and -540.66 pA respectively, and the inhibition rate on $I_{Nav}1.4$ was 42.72%, 49.50% and 52.32 % respectively with 4 mg/ml hydroalcoholic extracts. The $I_{Nav}1.4$ decreased from -2244.60, -5140.60 and -1133.90 pA to -0.00, 0.00 and 0.00 pA respectively, and the inhibition rate on $I_{Nav}1.4$ was 100.00%, 100.0% and 100.00 % respectively with 8 mg/ml hydroalcoholic extracts. The inhibition ratio of the 8 mg/ml group was obviously higher than the 4 mg/ml group ($P< 0.05$; $n=3$; Figure 2D1). This inhibitory effect of the hydroalcoholic extracts of G. inflata at 90% (v/v) on the $I_{Nav}1.4$, appeared within 10 min after initiating perfusion of the bath solution containing the hydroalcoholic extracts of G. inflata at 90% (v/v) (Figure 2D2). Once G. inflata was washed out, the inhibition effect of G. inflata on $I_{Nav}1.4$ was eliminated quickly (Figure 2A2, B2, C2 and D2). The inhibition effects of G. inflata on $I_{Nav}1.4$ is presented in Figure 2E.

3.4 Activation and inactivation kinetics, the recovery curve, frequency and concentration dependent inhibition effect of the hydroalcoholic extracts of G. inflata at 50% (v/v) on Nav1.4

Figure 3A presents the effect of a 3 mg/mL hydroalcoholic extracts of G. inflata at 50% (v/v) on the current voltage relationship of $I_{Nav}1.4$. The study shows that the 3 mg/ml hydroalcoholic extracts of G. inflata at 50% (v/v) significantly restrained the $I_{Nav}1.4$ peak current and decreased the $I_{Nav}1.4$ by more than 40% as compared the control. However, G. inflata did not have an effect on the reversal potential and activation property of $I_{Nav}1.4$ (Figure 3A). The research showed that the treatment with 3 mg/ml hydroalcoholic extracts of G. inflata at 50% (v/v) did not have an effect on the activation curve of Nav1.4. The V_h values (in mV) were -30.1 ± 2.1mV for pre-treatment and gave values of -35.4 ± 3.3mV for post-treatment, ($P >0.05$). The K values were 5.8 ± 0.9mV for pre-treatment and 6.2 ± 1.3mV for post-treatment, ($P> 0.05$; Figure 3B1). It did not change the V_h values (in mV) or the K values.

The treatment with 3 mg/mL hydroalcoholic extracts of G. inflata at 50% (v/v) had the following functions: the Nav1.4 inactivation curve shifted to the hyperpolarization side and the voltage of the half-maximal inactivation shifted to the hyperpolarization side by 21.8 mV as compared the control (Figure 3 B2). The research showed that the 3 mg/mL hydroalcoholic extracts of G. inflata at 50% (v/v) significantly affected the Nav1.4 inactivation curve, which changed the V_h values (in mV) from -63.7 ± 2.3mV to -84.7 ± 2.8mV ($P< 0.05$) and the K values from 6.5 ± 0.8 mV to 6.4 ± 1.1 mV ($P> 0.05$). Moreover, the 3 mg/mL hydroalcoholic extracts of G. inflata at 50% (v/v) significantly affected the Nav1.4 recovery curve from steady-state inactivation and increased the recovery time from 14.5 ± 1.3 ms for pre-treatment, to 37.6 ± 2.6 ms for post-treatment ($P< 0.05$; Figure 3 B3).

The use-dependency of 3 the mg/mL hydroalcoholic extracts of G. inflata at 50% (v/v) was tested under the following procedure: first, the membrane potential was held at -90 mV, and then depolarized to -10 mV and holden for 50 ms. The use-dependency was recorded at the frequencies of 1, 3 and 10 Hz. It was found that the 3 mg/ml hydroalcoholic extracts of G. inflata at 50% (v/v) presented a weak use-dependency ($P >0.05$; Figure 3 C1, C2 and C3). Hence, the concentration of 3 mg/ml did not present a frequency dependent inhibition rate of the hydroalcoholic extracts of G. inflata at 50% (v/v).

The concentration dependency of the hydroalcoholic extracts of G. inflata at 50% (v/v) on the Nav1.4 current was tested under a two-pulse procedure. From a holding potential of -120 mV, the first depolarizing test pulse was followed by a hyperpolarizing conditioning of the inter-pulse to half the inactivation voltage (8 s interval), followed by 20 ms of the recovery period at -120 mv. Then the second depolarizing test pulse of 0 mV for 20 ms. The inhibition of the Nav1.4 current at the resting state (TP1) and half inactivating state (TP2) were then calculated (Figure 3 D1). The IC50 of the hydroalcoholic extracts of G. inflata at 50% (v/v) was 2.12 mg/ml at the resting state (Figure 3 D2 and D3) and 0.99 mg/ml at the half inactivating state (Figure 3 D4 and D5).

3.5 Effect of eleven compounds of G. inflata on Nav1.4

The study showed that the inhibitory effects of eleven chemical compounds of G. inflata on $I_{Nav}1.4$ at the concentration of 10 μmol/L. Treatment with echinatin, isoliquiritigenin, glycyrrhizic acid, liquiritin, formononetin, liquiritigenin, glycyrrhetinic acid and neoisoliquiritin displayed inhibition rates on $I_{Nav}1.4$, and decreased the $I_{Nav}1.4$ by (33.20 ± 1.61)%, (39.98 ± 4.55) %, (20.54 ± 4.82)%, (22.62 ± 0.30)%, (18.63 ± 0.53)%, (19.89 ± 3.15)%, (14.90 ± 1.98)% and (15.02 ± 3.24)% respectively. By contrast, neoliquiritin, liquiritin apioside, and isoliquiritin had almost no inhibitory effect on $I_{Nav}1.4$ (Table 3; and Figure 4). In addition, the structural formulas of the eleven compounds are shown in Figure 5.

Table-3: Effect of eleven chemical compounds of G. inflata on peak current of Nav1.4

Compounds	Pre- treatment（pA）	Post- treatment（pA）	Inhibition（%）
isoliquiritigenin	-5187.75±439.04	-3128.05±597.43*	39.98±4.55
echinatin	-1206.61±865.77	-796.18±550.87*	33.20±1.61
liquiritin	-1427.88±1182.59	-1107.36±921.14*	22.62±0.30
glycyrrhizic acid	-3748.10±2095.58	-3049.85±1920.86*	20.54±4.82
liquiritigenin	-631.98±68.92	-507.81±83.40*	19.89±3.15
formononetin	-1334.00±721.54	-1088.15±597.15*	18.63±0.53
neoisoliquiritin	-2231.96±1874.31	-1939.55±1694.87*	15.02±3.24
glycyrrhetinic acid	-4123.25±987.90	-3495.05±725.14*	14.90±1.98
liquiritin apioside	-1497.16±789.76	-1387.76±681.00	6.26±2.80
isoliquiritin	-2946.90±853.05	-2741.00±627.06	6.13±4.17
neoliquiritin	-1817.06±1413.85	-1728.81±1298.52	3.42±2.60

* $p < 0.05$, n=3

4. DISCUSSION

Herein, we investigated the inhibitory effect of the hydroalcoholic extracts of G. inflata on $I_{Nav}1.4$. The results showed that the 4 and 8 mg/ml hydroalcoholic extracts of G. inflata at 30% and 50% (v/v) had a more potent inhibitory effect on $I_{Nav}1.4$, with inhibition rates of 96.70 – 100 percent. Further, the 8 mg/ml hydroalcoholic extracts of G. inflata at 70% and at 90% (v/v) also had a more potent inhibitory effect on $I_{Nav}1.4$, with an inhibition rate of 100 % each. However, the inhibition rate decreased to 32.14 % and 48.18% at a concentration of 4 mg/ml respectively. Overall, the hydroalcoholic extracts of G. inflata displayed acceptable inhibition of $I_{Nav}1.4$. Compared with G. uralensis, the 8 mg/ml groups displayed similar inhibitory effect at 30%, 50% and 70% (v/v), with the inhibition rates of 96.94 – 100 % (RSD % = 1.07%). However, the 8 mg/ml groups had different inhibitory effects at 90% (v/v), with inhibition rates of (100.0 ± 0.04)% and (16.63 ± 4.00)% respectively. The 4 mg/ml group also had quite different inhibition rates from each other at 30%, 50%, 70% and 90 % (v/v), which were respectively (100.0 ± 0.12)%, (96.70 ± 1.86)%, (32.14 ± 3.84)% and (48.18 ± 2.85)% with G. inflata, and (77.29 ± 3.00)%, (31.98 ± 9.00)%, (100.00 ± 1.00)% and (2.42 ± 1.00)% with G. uralensis. Judging from the results of the experiment described in this article, the inhibitory effect of Nav1.4 of G. inflata was superior to that of G. uralensis.

To further research its inhibitory effect on $I_{Nav}1.4$ and the channel dynamics effect, 3 mg/ml of the hydroalcoholic extracts of G. inflata at 50 % (v/v) was also studied. We discovered that it did not have the effect of altering the shape of the reversal potential or the I-U curve, nor did it affect the Nav1.4 activation curve. Thus, the hydroalcoholic extracts of G. inflata at 50% (v/v) exerted a non-significant inhibition ratio on the activation state of Nav1.4. Nevertheless, it significantly affected the Nav1.4 recovery curves and inactivation, and it shifted the inactivation curve to the hyperpolarization side and clearly increased the recovery time of Nav1.4 from the inactivation state. We concluded that the hydroalcoholic extracts of G. inflata at 50% (v/v) altered the inactivation characteristics of Nav1.4 and increased the recovery time of Nav1.4 from the inactivating to the resting state. Compared with G. uralensis, the two different drugs showed basically the same effect on ion channel dynamics.

In our previous experiments, we studied the hydroalcoholic extracts of G. uralensis. Eleven compounds of the hydroalcoholic extracts of G. uralensis were chosen to explain their different degrees of inhibition with regard to $I_{Nav}1.4$. The results presented that treatment with 10 μmol/l echinatin, isoliquiritigenin, glycyrrhizic acid and liquiritin decreased the $I_{Nav}1.4$ by more than 20% as compared the control; the values were respectively (33.20 ± 1.61)%, (39.98 ± 4.55)%, (20.54 ± 4.82)% and (22.62 ± 0.30)% . The remaining seven chemical compounds displayed a lower inhibition rate. Thus, echinatin, isoliquiritigenin, glycyrrhizic acid and liquiritin were selected as marker substances in inhibiting $I_{Nav}1.4$. Based on the above observations, further studies were carried out for quantitative analysis of the four components of the 4 and 8 mg/ml hydroalcoholic extracts (v/v, 30%, 50%, 70%, and 90 %) of G. inflata. It was subsequently found that glycyrrhizic acid reached a maximal concentration of 182.71μM, while echinatin had the lowest concentration, with a maximal value of only 0.59 μM.

Traditional Chinese medicine theory holds a synergistic effect between the chemical compounds of Chinese medicine, leading to a treatment effect [24-26]. The four chemical compounds may have cooperative effects and might finally produce a marked effect in the inhibition of Nav1.4 VGSCs currents, or there may be many other chemical compounds besides these eleven compounds that have been already researched previously. Moreover, one or more of these compounds might have the inhibition effect on $I_{Nav}1.4$ and work in coordination with each other to produce therapeutic effect. So it is hard to consider which specific chemical compound is most responsible for the therapeutic effect of the hydroalcoholic extracts of G. inflata on Nav1.4 VGSCs currents under the experimental conditions

described herein. Nonetheless, researchers still conclude that echinatin, isoliquiritigenin, glycyrrhizic acid and liquiritin play a key role in blocking-up Nav1.4 VGSCs currents.

In summary, $I_{Nav}1.4$ was inhibited by the hydroalcoholic extracts of G. inflata and in a dose-dependent manner. More researches are needed to resolve the electrophysiological mechanisms of the inhibitory effect of the hydroalcoholic extracts of G. inflata on Nav1.4 VGSCs. Moreover, eight other a-subunits (Nav1.1 - 1.3; and 1.5 - 1.9) and four different β-subunits (β1 - β4) should be researched. Meanwhile, the inhibition effect of Glycyrrhiza glabra on $I_{Nav}1.4$ should be studied to analyze the relationship between the three different species of licorice. The researches will enhance the scientific elucidation of the significance of the different sources of licorice in the treatment of many diseases and conditions.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Figures

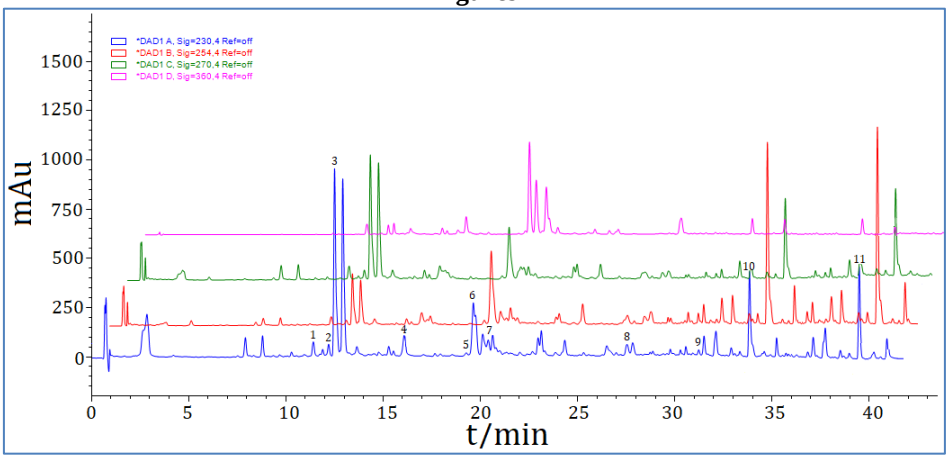

Fig-1: **UPLC-DAD chromatogram of the hydroalcoholic extracts of G. inflata at 50% (v/v) at wavelengths of 230 nm, 254 nm, 270 nm and 360 nm**

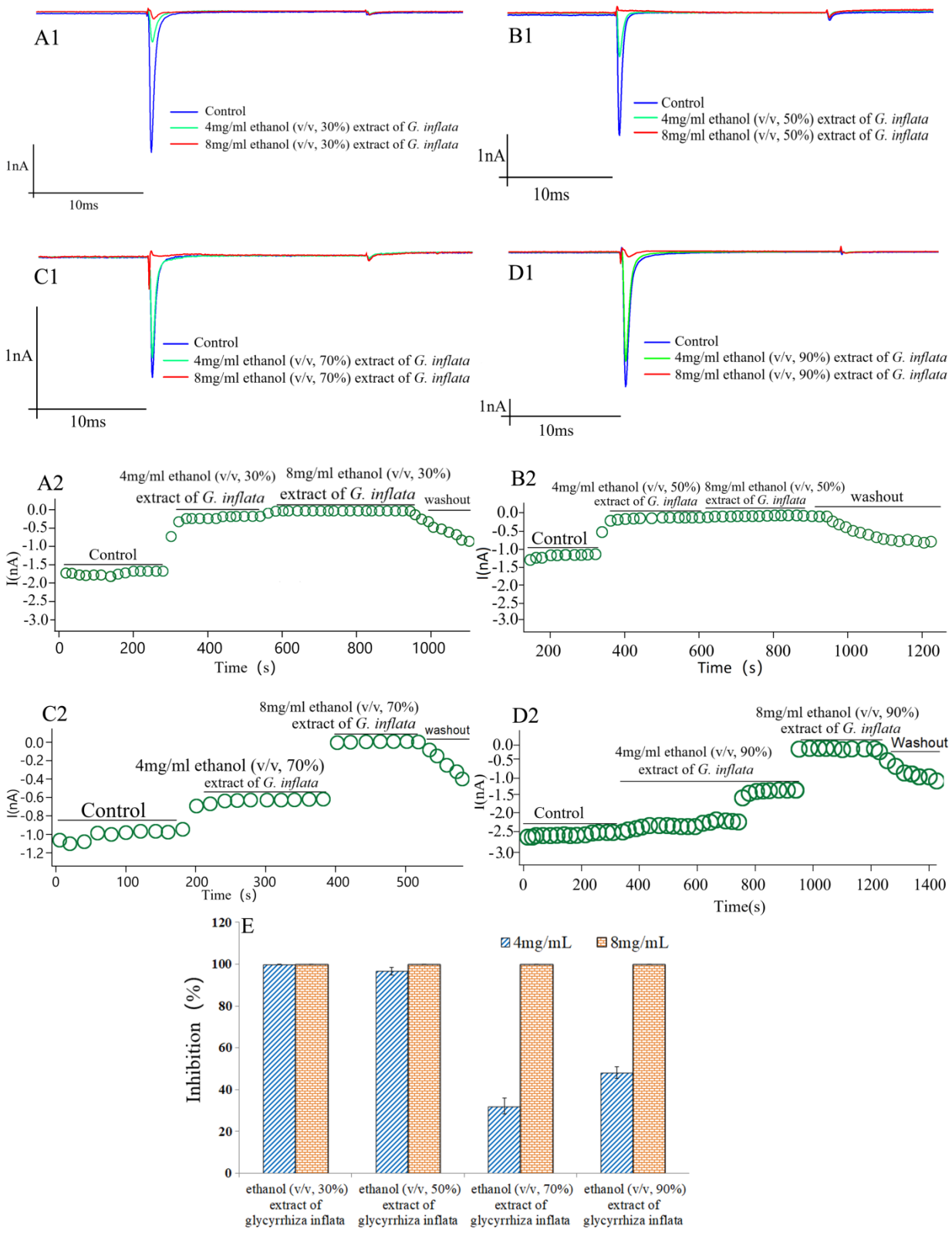

Fig-2: **Effect of hydroalcoholic extracts of G. inflata on $I_{Nav}1.4$**

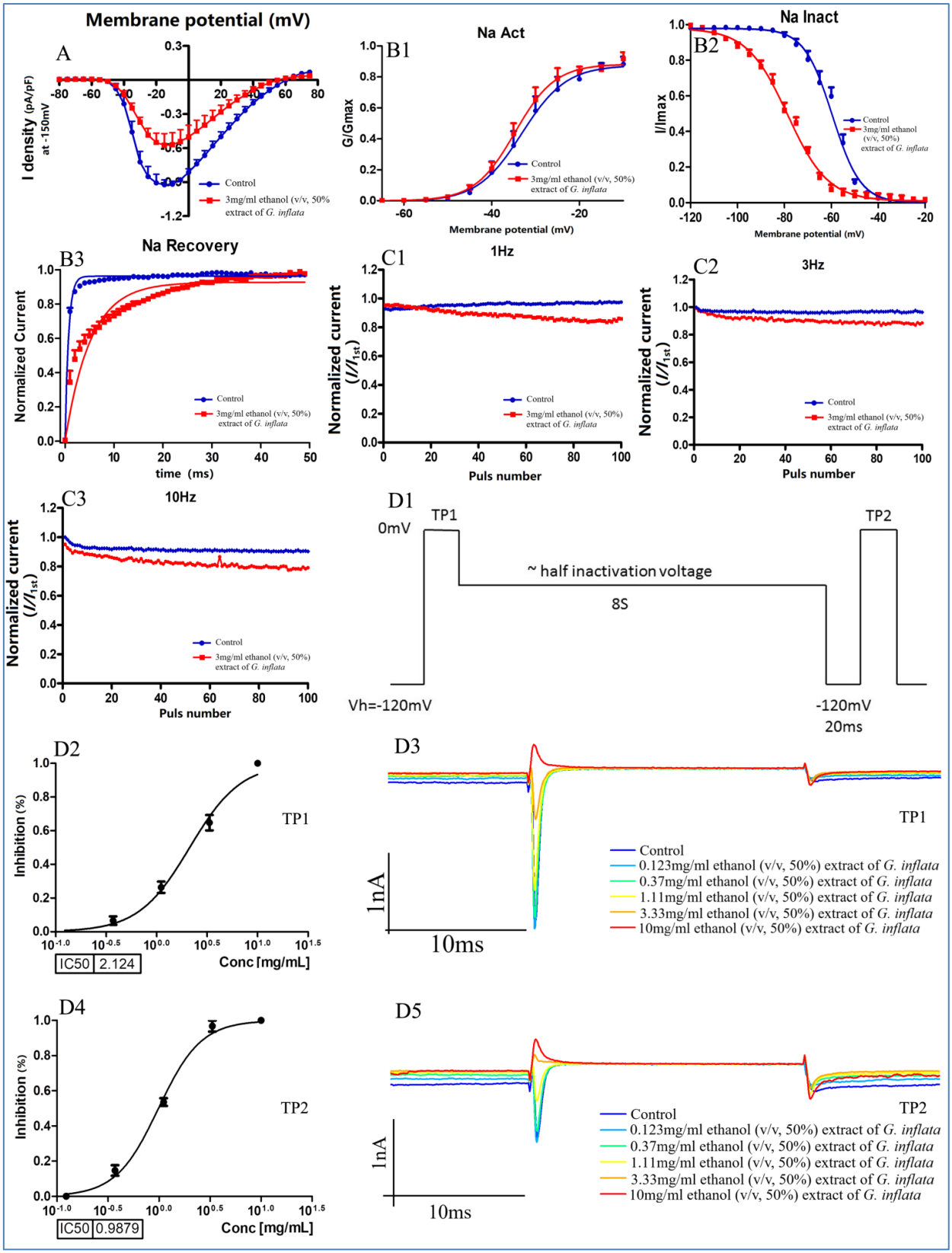

Fig-3: Effect of the hydroalcoholic extracts of G. inflata on $I_{Nav}1.4$ and kinetics

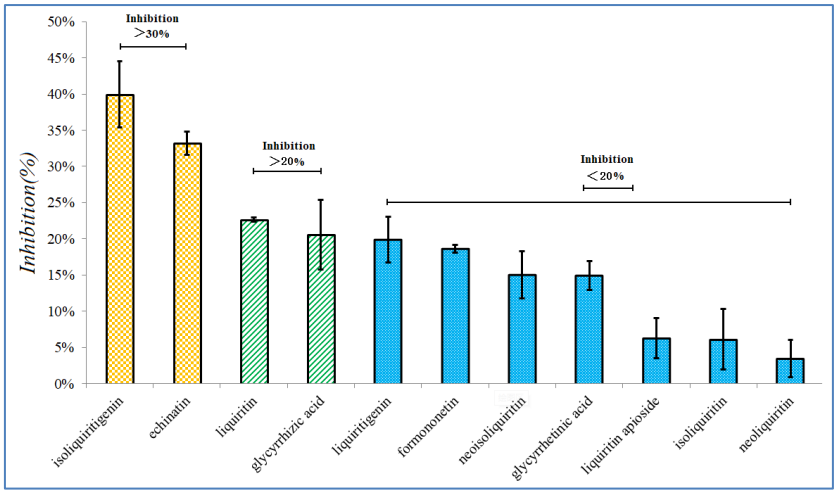

Fig-4: **Inhibition of the eleven compounds on $I_{Nav}1.4$**

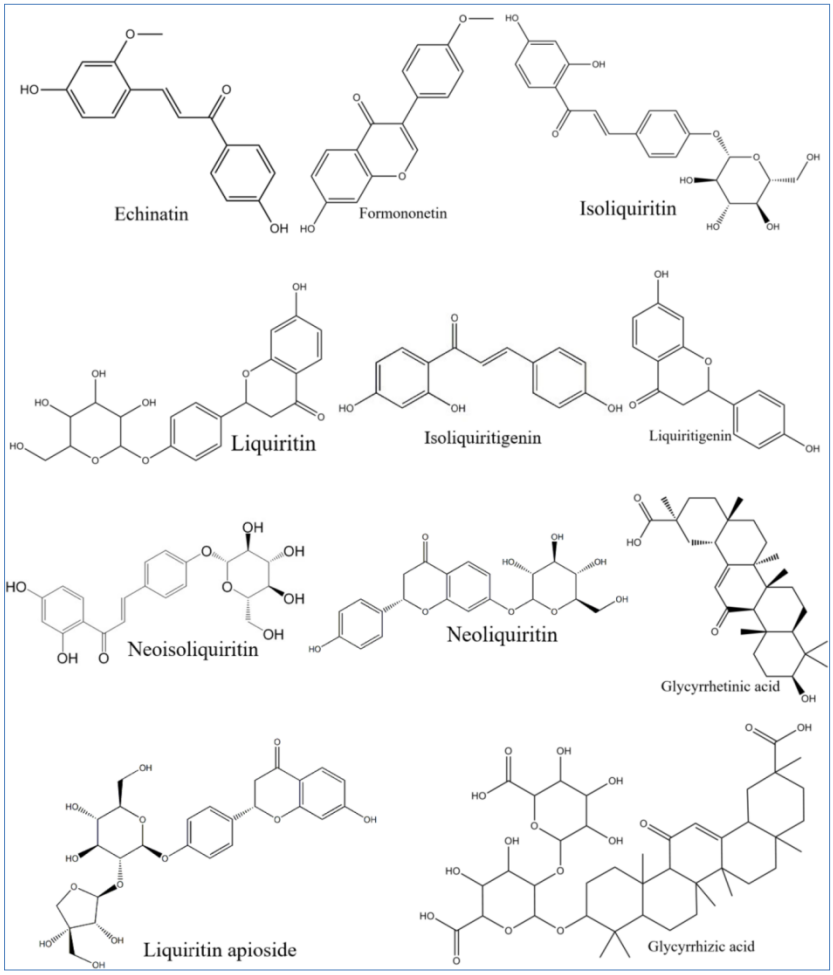

Fig-5: Structures of the eleven studied compounds

Figure Legends

Figure 1. UPLC-DAD chromatogram of the hydroalcoholic extracts of G. inflata at 50% (v/v) at wavelengths of 230, 254, 270 and 360 nm. The vertical axis represents the response value (mAu) and the horizontal axis represents the retention time (in mins). 1. Formononetin; 2. liquiritin apioside; 3. liquiritin; 4. neoliquiritin; 5. neoisoliquiritin; 6. isoliquiritin; 7. liquiritigenin; 8. echinatin; 9. isoliquiritigenin; 10. glycyrrhizic acid; and 11. glycyrrhetinic acid.

Figure 2. Effect of hydroalcoholic extracts of G. inflata on $I_{Nav}1.4$. A1, B1, C1, D1) $I_{Nav}1.4$ recorded from CHO cells expressing human Nav1.4 VGSCs before G. inflata treatment (control), under treatment, and after washing out G. inflata. A2, B2, C2, D2). The

time course of inhibiting the $I_{Nav}1.4$ when evoked by depolarization from a holding potential of −120 mV to a test potential of +10 mV by sequential exposure to G. inflata is shown. The amplitude of the $I_{Nav}1.4$ was measured every 20 s. The ordinate shows the $I_{Nav}1.4$ relative to the control current during exposure of the cell to G. inflata and during wash-out of G. inflata. E) The inhibitory effect of the hydroalcoholic extracts of G. inflata on $I_{Nav}1.4$. A1, A2: 4 mg/ml and 8 mg/ml hydroalcoholic extracts of G. inflata at 30% (v/v). B1, B2: 4 mg/ml and 8 mg/ml hydroalcoholic extract of G. inflata at 50% (v/v). C1, C2: 4 mg/ml and 8 mg/ml hydroalcoholic extracts of G. inflata at 70% (v/v). D1, D2: 4 mg/ml and 8 mg/ml hydroalcoholic extracts of G. inflata at 90% (v/v). E: 4 mg/ml and 8 mg/ml hydroalcoholic extracts of G. inflata at 30%, 50%, 70% and 90% (v/v).

Figure 3. Effect of the hydroalcoholic extracts of G. inflata on $I_{Nav}1.4$ and kinetics. A) The voltage dependent effect of G. inflata on $I_{Nav}1.4$ density. B1) Effect of 3 mg/ml hydroalcoholic extracts of G. inflata at 50% (v/v) on steady-state activation kinetics of $I_{Nav}1.4$. Data was obtained from the I-U relationship. B2) Effect of 3 mg/ml hydroalcoholic extracts of G. inflata at 50% (v/v) on steady-state inactivation kinetics of $I_{Nav}1.4$. The steady-state inactivation curves of $I_{Nav}1.4$ were obtained using a double-pulse protocol; a one second conditioning prepulse depolarized to various potentials (from -90 mV to 0 mV, holding potential at -80 mV) was followed by a 200 ms test pulse to 0 mV. B3). Effect of 3 mg/ml hydroalcoholic extracts of G. inflata at 50% (v/v) on the recovery curve of $I_{Nav}1.4$ from steady-state inactivation. Using a standard two-pulse protocol, a 200 ms prepulse to 0 mV from the holding potential of -40 mV was followed by various recovery durations and then by a test pulse to 0 mV for 200 ms. C1, C2 and C3) The use-dependent inhibition protocol: the membrane potential was held at -90mV, and then depolarized to -10 mV and maintained for 50 ms, then recorded at frequencies of 1, 3 and 10 Hz. D1, D2, D3, D4 and D5) The concentration-dependent protocol, (TP1:Resting state, TP2:Rnactivating state).

Figure 4. Inhibition of the eleven compounds on $I_{Nav}1.4$. The compounds all had certain inhibitory effects on Nav1.4. Four of them displayed relatively high inhibition rate (generally over 20 percent), and included the following compounds: isoliquiritigenin, echinatin, liquiritin, and glycyrrhizic acid, respectively.

Figure 5. Structures of the eleven studied compounds.

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