Oleamide in *Ipomoea* and *Dillenia* species and inflammatory activity investigated through ion channel inhibition

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ABSTRACT

Background: Oleamide is an essential substance for human health. So, the plants with high oleamide content are great sources for health care products.

Objective: This study is conducted to investigate the quality of oleamide in plants and test the bioactivity in the selected two studied species.

Methods: The three *Ipomoea* and five *Dillenia* species including *Ipomoea alba*, *Ipomoea aquatica* and *Ipomoea pes-caprae*, and *Dillenia indica*, *Dillenia obovata*, *Dillenia ovata*, *Dillenia parviflora* and *Dillenia pentagyna* were investigated for the quantity of oleamide by high-performance liquid chromatography. The biological activity test was conducted on the powder formulation of the chosen plants, *Dillenia ovata* and *Dillenia parviflora* at a ratio of 30:70, for anti-inflammatory activity *ex vivo* on a panel of molecular targets through ion channel inhibition including voltage-gated sodium channel, voltage-gated potassium channel, and the cardiac ion as human ether-a-go-go related gene.

Results: The results showed that the leaf extracts of *I. aquatica* and *D. ovata* gave the highest and subsequent oleamide quantity following 7.52 and 5.17 mg/g. Out of the *Dillenia* formulation which contained various compounds, oleamide showed the highest percentages of inhibition at 8.0-20.0%, and 6.2-14.2% in voltage-gated sodium channel, and voltage-gated potassium channel which had slightly lower values than the oleamide standard, and no effect as 0.0% value inhibition in the cardiac ion channel.

Conclusion: The *Dillenia* formulation exhibits anti-inflammatory activity without affecting the heart. Accordingly, the three studied *Ipomoea* and three studied *Dillenia* species may be used for the same activity as a single component or formulation with effective solvent for disease treatments.

Keywords: *Dillenia*, high-performance liquid chromatography (HPLC), *Ipomoea*, inflammatory activity, ion channel inhibition, oleamide

List of Abbreviations:

DMSO – dimethyl sulfoxide
GC-MS – gas chromatography-mass-spectrometry
hERG – human ether-a-go-go related gene
HPLC – high-performance liquid chromatography
LDL – low-density lipoprotein
TEVC – two-electrode voltage-clamp technique
VGSC – voltage-gated sodium channel
VGKC – voltage-gated potassium channel

INTRODUCTION

Phytochemicals and plants are very important for humans. They have been used to support human living, health, and well-being through various forms, including consumption as foods, functional foods, nutraceuticals, vegetables, and traditional medicine. Many modern drugs were developed from purified, synthetic, or modified forms of phytochemicals. Besides food, the use of the plants for consumption in human affiliated to their phytochemical content. γ-Sitosterol from the *Lagerstroemia* species has an antihyperglycemic activity [1]; and phytosterols, which consist of both plant sterols and plant stanols, help in lowering LDL-cholesterol concentrations [2].

Phytochemicals are also important ingredients in skin lightening products, which are included in cosmetics worldwide. Many types of skin lightening substances for example β-arbutin can be obtained from natural sources [3,4]. One more, oleamide is a great substance that occurs naturally in the body of animals, including humans, and accumulates in the cerebrospinal fluid during sleep deprivation. There is a long history of researches related to its functions. It was revealed that oleamide is a protective agent against scopolamine-induced memory loss and is suggested to be useful as a chemopreventive agent against Alzheimer's disease, it induces deep sleep, up-regulation of appetite, shows induced deep sleep activity, is not related to changes in blood pressure, heart rate, or body temperature [5-7]. Recently, one

of its functions was related to anti-inflammatory activities following disclosing. Oleamide shows an anti-inflammatory effect through inhibition of nuclear factor-kappa B activation in lipopolysaccharide-stimulated BV2 microglia [8]; has been used for the prevention and treatment of athero-sclerosis, thrombosis, arthritis, and cancer through its metabolic conversion into pros-taglandins, thromboxanes, and leukotrienes, can be used as a single ingredient treatment for inflammatory diseases [9]; is an endocannabinoid and displays anti-inflammatory activity via the cannabinoid-2 receptor [10].

So, the plant species containing oleamide are very important especially the edible plants. The two-plant genera, *Dillenia* and *Ipomoea* are of interest to the researchers. They have the potential to be used in many ways if additional studies are needed, such as exact amounts of the substance using HPLC or other methods and their biological activity. Some *Dillenia* and *Ipomoea* species are edible and used as traditional medicine. Thooptianrat et al. [7] reported that the nine *Dillenia* species including *D. ovata* and *D. parviflora* were studied by gas chromatography-mass-spectrometry (GC-MS), contained 18.05–75.60% oleamide, and the other components, squalene and vitamin E, discovered in high amounts, are nontoxic to normal human cells both in cytotoxic and genotoxic levels, thus may be safely applied for the treatment of Alzheimer's disease and other related conditions. *Ipomoea* species is a plant group, which has long been used for human living as cooking and vegetables named *I. alba, I. aquatica*. Phytochemical contents of some species as *I. pes-caprae, I. cairica* and some wild species have been used as traditional medicine for several treatments for example: treatment of inflammatory and analgesic processes, heated leaves are used for treating wound, skin infections, inflamed sores and stings from poisonous fish, manta-ray and insects, infusions have been recommended for treating hypertension, kidney ailments and decoctions to treat digestive disorders, colic, internal and external pain, dysentery, inflammations, fatigue, strain, arthritis and rheumatism, etc. Phytochemical contents also varied [11]. In the targeted plants, the most essential factor to examine before being used in human is plants' bioactivities testing. There are various methods, and ion channels that are alternative for the testing *ex vivo* on a panel of molecular targets, rather than on animals. The reliable ion channel protocol showed that 15% of the currently used drugs target ion channels [12]. There are several channel inhibitions, as voltage-gated sodium channels (VGSCs), voltage-gated potassium channels (VGKCs), human ether-a-go-go related gene (hERG chanel or Kv11.1 channel), that facilitate the various activities occurring in the cells. For example, VGSCs ($Na_v1.1$, 1.4, 1.5, 1.6 and 1.8) related to the report of Cummins *et al*. [13] revealed anesthetics of sodium channel blockers. Therefore, the objective of this study is to investigate

oleamide in *D. indica*, *D. obovata*, *D. pentagyna*, *D. ovata* and *D. parviflora*, *D. obovata* and *D. parviflora*, and *I. alba*, *I. aquatica* and *I. pes-caprae*, and finally conduct their bioactivity testing.

MATERIALS AND METHODS

Plant materials

The three *Ipomoea* and five *Dillenia* species including *I. alba, I. aquatica* and *I. pes-caprae*, and *D. indica*, *I. obovata*, *D. pentagyna*, *D. ovata* and *D. parviflora* were collected. The plants were identified by Professor Dr. Arunrat Chaveerach who is a proficient botanist.
Voucher specimens were stored at the Department of Biology, Faculty of Science, Khon Kaen University, Thailand. The voucher specimen numbers are A. Chaveerach 930 to A. Chaveerach 934 and A. Chaveerach 935 to A. Chaveerach 947 for *Dillenia* and *Ipomoea* species, respectively. The leaves were rinsed and air-dried for 2-3 days, then used for oleamide investigating and bioactivity testing.

Methods

1. Oleamide analysis and quantification from the studied samples by HPLC

1.1 Analysis of the rice bran oil plant extract

The dried leaves were finely ground, and 2 g of the powder was added with 10 ml rice bran oil (1:5). The solutions were incubated for 48 h in the dark at room temperature. Then, they were subsequently filtered with a thin cloth and filter paper. Thereafter, 1 ml of solution was added with 1 ml hexane (1:1), then incubated for 24 h in the dark at room temperature. The hexane solvent was then removed via a rotary evaporator (Rotavapor R-210; Buchi, Switzerland) at 800-1,000 mbar, 15°C, 600 rpm for 2 h. Dimethyl sulfoxide (DMSO, 100%) was added to the extracts using an equal amount of evaporated solvent. The 100% DMSO extracts were diluted to be 10% DMSO by deionized water and were analyzed by HPLC using a Shimadzu LC-20AD (Japan) model with a quaternary pump, PAD (SPD-M20A) detector, and column Inertsl ODS-3 C18, 4.6×250 mm, 5 microns (GL Sciences Inc.). The 100 µl sample was injected. The mobile phase consisted of two solutions, methanol: acetonitrile at a rate of 30:70. The elution was carried out at a flow rate of 1 ml/min. The detection wavelength was 202 nm.

1.2 Analysis of the methanol plant extract

A 2 g sample was ground into a powder, mixed with 10 ml methanol **(**HPLC grade**)** and filtered through filter paper. The filtrate was used for HPLC analysis using the identical protocol and instruments as mentioned in the topic 1.1.

1.3 Analysis of oleamide standard and calibration curve

Linearity for the oleamide standards (dissolved in methanol) derived from graph plotting between linear regression of the peak areas resulted from HPLC analysis and the three levels of concentration standards as, 0.0625, 0.125 and 0.250 and 0.500 mg/ml for the studied *Dillenia* species, 0.0625, 0.125, 0.250, 0.500 and 1.000 mg/ml for the studied *Ipomoea* species, to create a linear curve, calibration equation and correlation factor (R^2) by Microsoft Excel. The calibration equations were used for oleamide evaluation from the eight studied samples, rice bran oil (10% DMSO form for HPLC injection) and methanol *D. Indica*, *D. 5bovate*, *D. Pentagyna*, *D. Ovata* and *D. Parviflora*, and *I. Alba*, *I. Aquatica* and *I. Pes-caprae* leaf extract samples. The correlation factor was applied for the reliability of the calibration equation.

2. Biological activity testing of the plant extracts on ion channels

The extracts were tested by two-electrode voltage-clamp technique (TEVC) in whole cell of *Xenopus laevis* oocytes with VGSCs ($Na_v1.1$, 1.4, 1.6, 1.8), VGKCs ($K_v1.1$, $K_v10.1$, and cardiac ion channel (hERG or $K_v11.1$).

2.1 Preparation of plant extracts and standards

The leaf samples of *D. ovata* and *D. parviflora* species were rinsed and air-dried, then ground and mixed to create a formulation at a rate of 30:70. A 20 g sample formulation was mixed with 100 ml hexane and acetonitrile (analytical grade, Sigma-Aldrich, USA) for 72 h. The samples were filtered through a filter paper (Whatman No. 1), and the filtrates were solvent evaporated with a rotary evaporator (Rotavapor R-210, Buchi, Switzerland) at 40°C, 600 rpm for 1-2 h., viscous crude extracts were obtained. Dimethyl sulfoxide (DMSO), 100% was added to the extracts until completely dissolved and diluted to be 0.5% DMSO with ND-96 solution (96 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, adjusted pH to 7.5 with NaOH) for experimenting with ion channels.

cis-Oleamide standard (Sigma-Aldrich, Belgium) was dissolved in 0.5% DMSO in ND-96 solution to prepare a 20 mM stock solution and stored at -20°C until required.

2.2 Insertion of recombinant receptors (ion channels) into oocytes

Stage V-V1 oocytes from female *Xenopus laevis* were received from the laboratory of Toxicology and Pharmacology, University of Leuven, Leuven, Belgium and stored in ND-96 solution mixed with gentamycin sulphate (1.25 mL) and theophylline (90 mg) at 16°C until required.

The cDNA encoding for studying channels was transformed in *Escherichia coli*. After isolation and linearization of plasmid containing cDNA, the cDNA was transcribed into cRNA by Rneasy MinElute Cleanup (Qiagen 74204) transcription kit and stored at -80°C until required. Each of the selected oocytes was injected with 30-50 ng of each cRNA. The injected oocytes were incubated in ND-96 solution at 16°C for 1-4 days depending on ion channel for enough cRNA expression. The cells were used for ion channel inhibition testing by electrophysiological recording.

2.3 Electrophysiological recording

The resistance oocyte electrodes were prepared between 0.3-0.5 MΩ and filled with 2 M KCl. A frequency of 2 Hz was used for the experiment. The cells were voltage-clamped using TEVC with voltage-gated ion channels as VGSCs, VGKCs and hERG. The prepared extracts and standard solutions were added into the oocyte separately, each at 3 μL. Each experiment was performed in triplicate cells. The voltage dependence of I_{Na} was determined from -90 mV to 65 mV in 5 mV increments. Also, the voltage dependence of I_K was elicited from -50 mV to 65 mV in 5 mV increment. All values were presented as means ± standard error. All data was analyzed by Clampfit 10.7 (Molecular devices, USA) and Origin 9.0 software (Originlab, USA).

RESULTS

The major component oleamide was measured as their released concentration (mg/mL) and amount (mg) by an HPLC. Separately, *D. indica*, *D. obovata*, *D. pentagyna*, *D. ovata* and *D. parviflora* were extracted with rice bran oil, and *I. alba*, *I. aquatica* and *I. pes-caprae* were extracted with rice bran oil and methanol. The extracted oleamide substance was found in all studied species indicating by peak areas compared to the oleamide standard, and the control, 10% DMSO was also, injected and released peak areas (Figures 1, 2). Linearity equation of the oleamide standard was derived from a graph of the peak areas and concentrations, produced the calibration equation, $y = 10^7x$-107662 and correlation coefficient (R^2), = 0.9989 of the studied *Dillenia* species; and y = 829034x + 42642 and correlation coefficient (R^2), = 0.9996

of the studied *Ipomoea* species, where y is the peak area. Subsequently, oleamide substance in the eight studied species was detected and evaluated following detected peak area characteristics, the retention time, and the calibration equations, sample weights for extraction and final extract volumes (ml). The results were declared for two choices as amount and concentration.

The studied *Dillenia* species were extracted with a rice bran oil solvent, oleamide amounts vary between 1.01 and 5.17 mg/g; and the concentrations are 0.326 to 2.249 mg/ml. The two methanol and rice bran oil solvents were used for extraction in the studied *Ipomoea* species, oleamide amounts are 1.10 to 14.47 mg/g and the concentrations are 0.12 to 4.82 mg/ml. These amounts and concentrations data are illustrated in Table 1.

In whole cell by TEVC, 100 µM of the oleamide standard show inhibitory activities against four VGSCs at 13.6 to 37.2%, and two VGKCs at 15.8 to 30.0%, and non-inhibitory activity to hERG channel showing by the graphs of inhibition effects in Figure 3.

The *Dillenia* formulation hexane and acetonitrile extracts resulted in different percentages of inhibition on VGSCs channel, 6.2-14.2%; and on VGKCs channel, 10.1-20.0%, with non-inhibition on hERG channel (Table 2, Figures 4-5).

Discussion

The rice bran oil *Dillenia* extracts revealed high percentages of oleamide as 1.01-5.17 mg/g (*D. indica-D. ovata*) leaves. According to Thooptianrat *et al*. [7], *Dillenia* hexane extracts contained oleamide, which has the same non-polar solvent and same plant genus.

While as methanol solvent extract showed high percentages of oleamide in all studied *Ipomoea* species as *I. alba, I. aquatica* and *I. pes-caprae*. Remarkably, with rice bran oil solvent, *I. pes-caprae* released the highest quantity and concentration as 7.52 mg/g leaf sample and 1.71 mg/ml (Table 2), which is correct because the chemicals in the plant are not in a single or purified molecule, but in group. So, phytochemical screening should be examined by both polar and non-polar solvents.

However, in the case of methanol *Ipomoea* extract, it is needed to be consumed by extracted form, the solvent can be cleared by evaporation and then gradually rice bran oil can be added to it. For more safety, ethanol solvent should be used instead. Soft mixed capsules of *D. ovata* and *I. aquatica* in rice bran oil are very interesting to form as they contain much useful phytochemicals in a plant. Supported by one more study, *D. ovata* was investigated to have no toxicity in normal human both at the cell and DNA levels [7]. The soft capsule may be used for Alzheimer's treatment due to its oleamide component [6], in addition with its anti-

inflammatory activity [9,10]. Moreover, the other chemicals in the extract may be beneficial for human body as functional foods or nutraceuticals.

In the research, the whole extract should be standardized biologically for treating distinct clinical conditions [14]. However, the reliable ion channel protocol, which shows 15*%* of currently used drugs targeting ion channels [12] were used here to test overall pharmacological activity of the two *Dillenia* species created formulation. These plant extracts are rich in useful bioactive compounds as oleamide are attractive sources for new leads in drug discovery through the ion pathway. Unfortunately, the research team failed to determine the optimum condition to test the effect of oleamide for induced sleep, memory recovery, anti-Alzheimer's, reduced stress and upregulated appetite in elderly in a research timing, but succeeded in activity testing following ion channels of VGSCs ($Na_v1.1$, 1.4, 1.6 and 1.8), VGKCs ($K_v1.1$, $K_v10.1$) and hERG channels exhibit anti-inflammatory activity. These researches are the result of plant formulation related to the report revealing anesthetics of sodium channel blockers. It is related to the anesthetics, reducing both inflammatory and neuropathic pain [13], in addition to chronic pain, which is a significant health problem [15]. Furthermore, $Na_v1.8$ channel related to these mentioned activity [15] has a high percentage inhibition to 14.2% compared to the oleamide standard at 37.2%. Additionally, the two studied plants as the formulation have $K_v1.1$ activity as may contribute to the intrinsic function of the heart [16], and $K_v10.1$ activity which has a lot more biological information as cancer biology, an early marker in tumor formation useful for tumor diagnosis and therapy [17].

CONCLUSION

Probably, the three studied *Ipomoea* and five *Dillenia* species may be used as a single or formula productions for disease treatments following oleamide containing and ion channel activity testing with respect to cardiac safety. All scientific data resulted were benefitted to the pathway of safe usage in human health as functional foods, nutraceuticals, and natural medicines.

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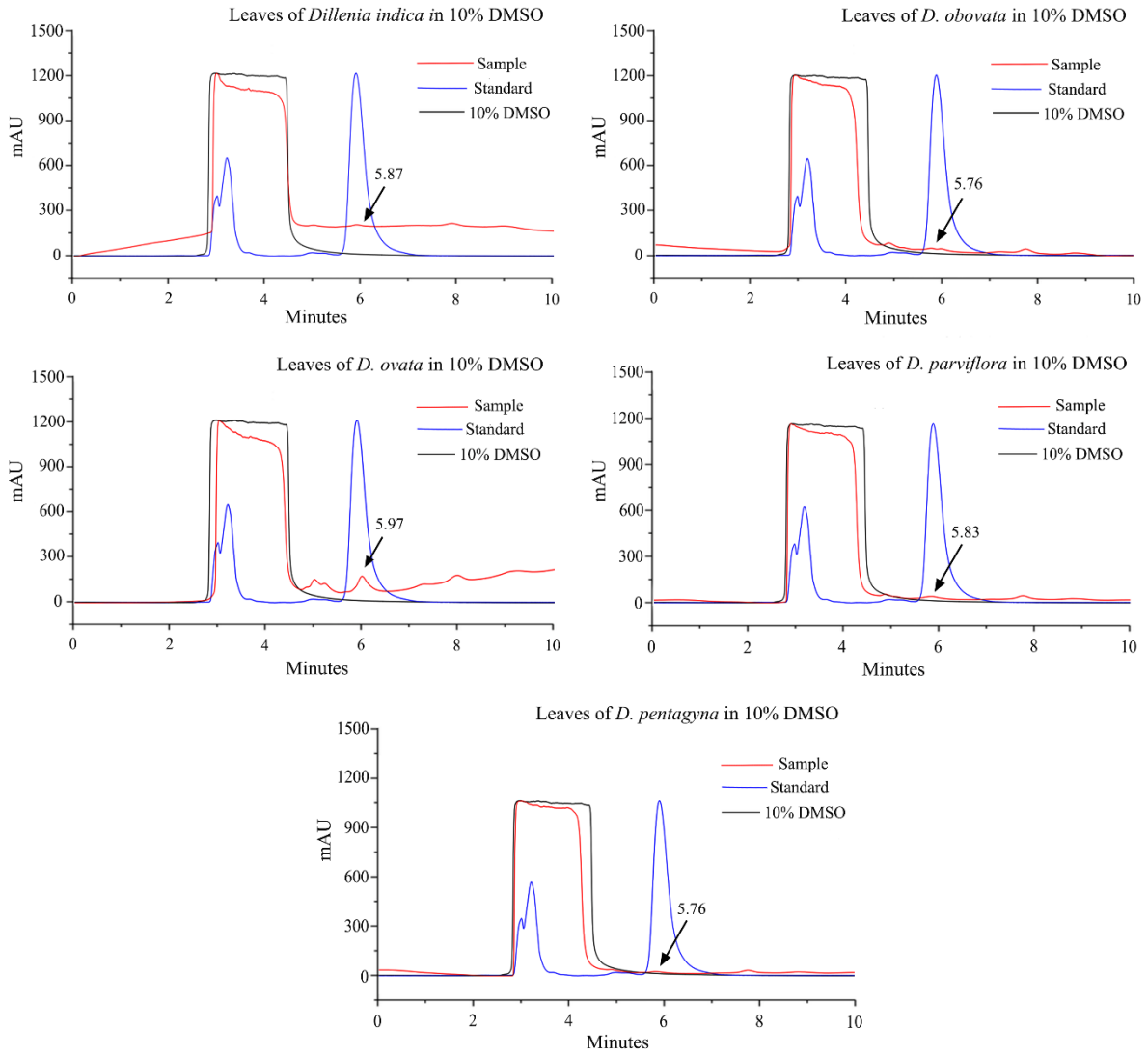

Figure 1 HPLC chromatograms showing peaks of oleamide standard, oleamide in the *Dillenia indica*, *D. obovata*, *D. ovata*, *D. parviflora* and *D. pentagyna* species rice bran oil with subsequent 10% DMSO extraction

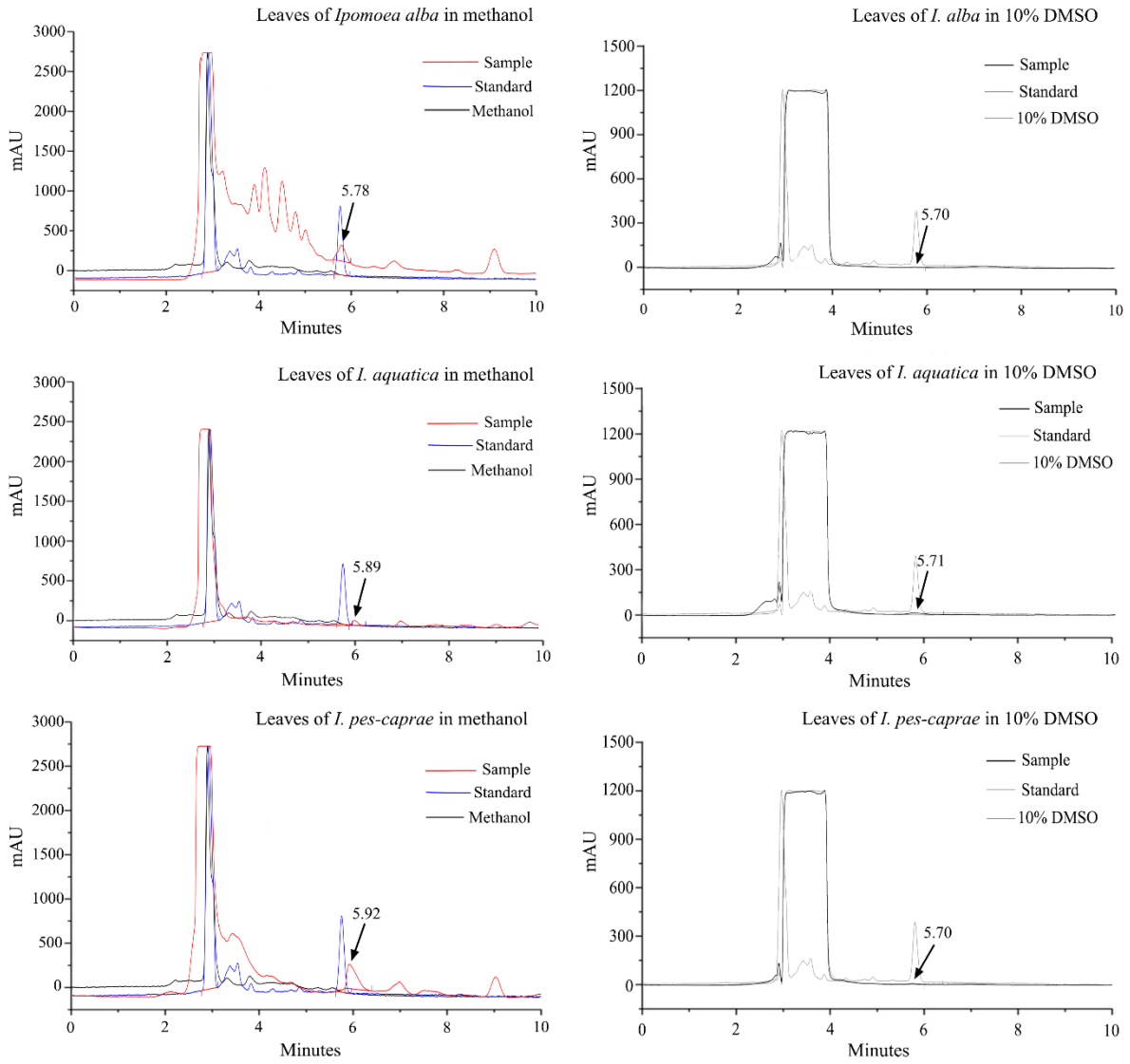

Figure 2 HPLC chromatograms showing peaks of oleamide standard, oleamide in the *Ipomoea alba*, *I. aquatica* and *I. pes-caprae* species extracted with methanol solvent and rice bran oil with subsequent 10% DMSO extraction

Table **1** The sample leaf extracts with methanol and rice bran oil solvents, and the results of HPLC analysis including peak area, concentration and amount of oleamide

Plant	Sample (g): Solvent (ml)		Filtrate extract volume (ml)		Peak area (mAU)		Oleamide concentration (mg/ml)		Oleamide amount (mg/g)	
	Methanol	Rice bran oil	Methanol	Rice bran oil	Methanol	Rice bran oil	Methanol	Rice bran oil	Methanol	Rice bran oil
Dillenia indica	N/A	10:50	N/A	31	N/A	218240	N/A	0.326	N/A	1.01
D. obovata	N/A	6:30	N/A	18	N/A	266789	N/A	0.374	N/A	1.12
D. ovata	N/A	10:50	N/A	23	N/A	2141590	N/A	2.249	N/A	5.17
D. parviflora	N/A	10:50	N/A	30	N/A	307277	N/A	0.415	N/A	1.24
D. pentagyna	N/A	9:45	N/A	40	N/A	154651	N/A	0.262	N/A	1.17
Ipomoea alba	2:10	20:100	2	45	2329171	15538	2.76	N.D.	2.76	N.D.
I. aquatica	2:10	15:75	3	44	652919	184316	0.74	1.71	1.10	7.52
I. pes-caprae	2:10	20:100	6	38	4041169	52639	4.82	0.12	14.47	0.65

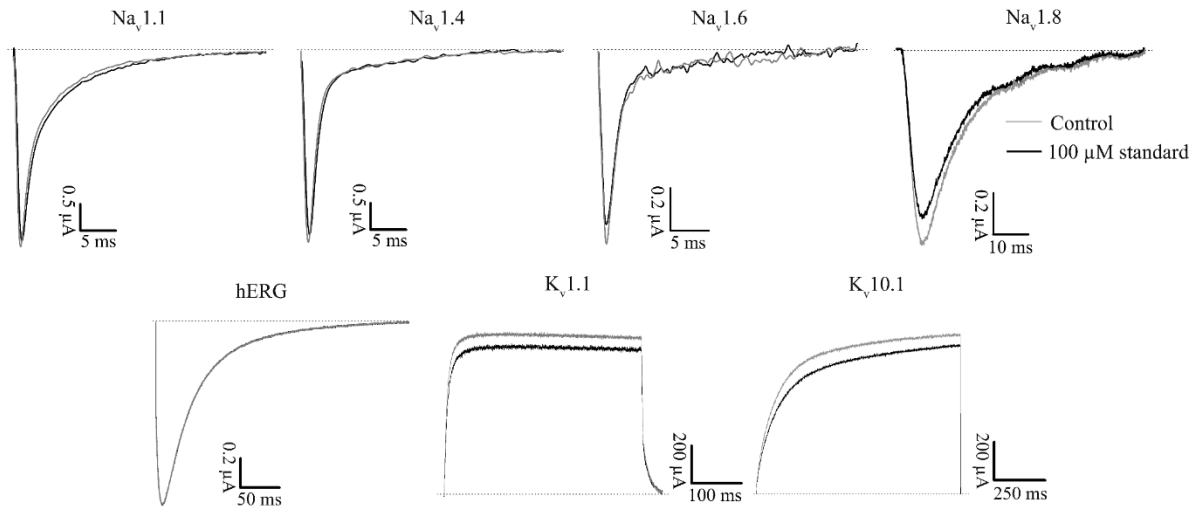

Figure 3 Inhibition effect of *cis*-oleamide standard on voltage-gated sodium channels ($Na_v1.1$, 1.4, 1.6 and 1.8), voltage-gated potassium channels ($K_v1.1$ and K_v 10.) and hERG channels**.**

Table **2** Percentage inhibition on voltage-gated ion channels (VGSCs: $Na_v1.1$, 1.4, 1.6, 1.8; VGKCs: $K_v1.1$, 10.1 and hERG) of oleamide standard and the two hexane and acetonitrile *Dillenea* leaf extract formulation (*D. ovata* and *D. parviflora* at a rate 30:70)

Voltage-gated ion channels	% Inhibition of the standard on ion channels	% Inhibition of D2 on ion channels	
		Hexane	Acetonitrile
$Na_v1.1$	13.6	8.0	13.1
$Na_v1.4$	19.4	14.0	6.2
$Na_v1.6$	21.9	7.4	10.2
$Na_v1.8$	37.2	12.3	14.2
$K_v1.1$	15.8	20.0	10.1
$K_v10.1$	30.0	17.2	12.1
hERG	0.0	0.0	0.0

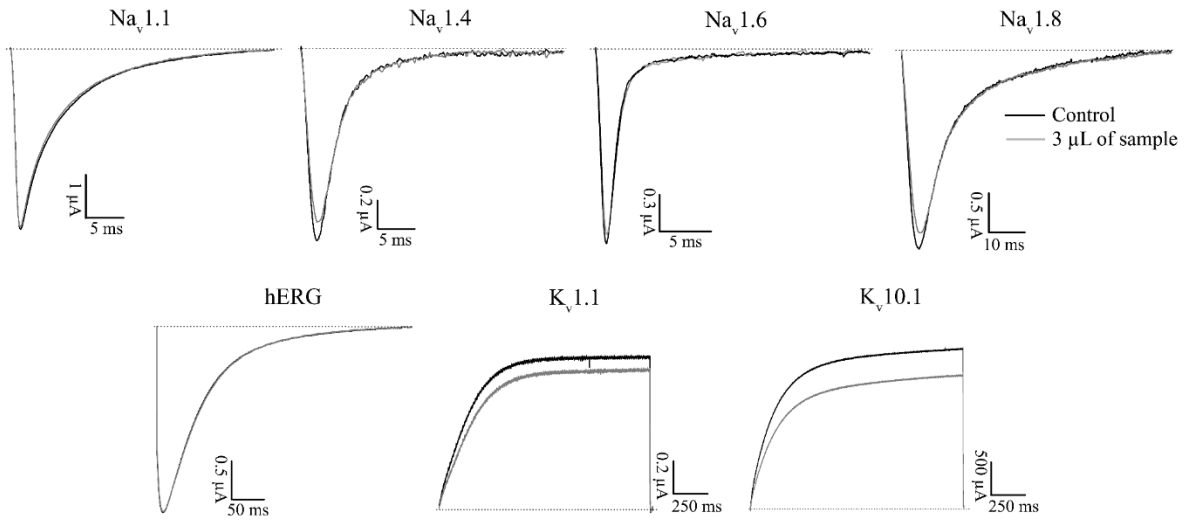

Figure 4 Inhibition effect of voltage-gated sodium channels, VGSCs (Na_v1.1, 1.4, 1.6 and 1.8); voltage-gated potassium channels, VGKCs (K_v1.1 and 10.) and hERG channels after treated with hexane D2 leaf extracts**.**

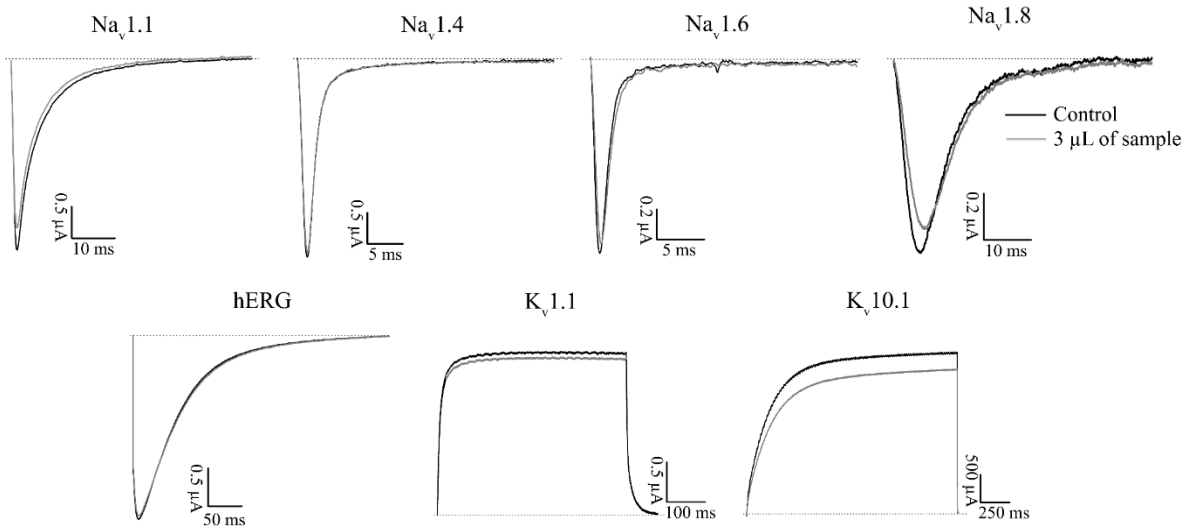

Figure 5 Inhibition effect of voltage-gated sodium channels, VGSCs (Na_v1.1, 1.4, 1.6 and 1.8); voltage-gated potassium channels, VGKCs (K_v1.1 and 10.) and hERG channels after treated with acetonitrile D2 leaf extracts**.**