MSc Food Safety

Master's Thesis

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The cytotoxicity of prenylated phenolics towards proliferating Caco-2 cells and their recovery efficiency

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Abstract

Prenylated phenolics are natural compounds found in various plants. In previous studies, they showed potential benefits to human health, such as used as phytoestrogens supplement and antibacterial drugs. Their prenylated side chains induce a higher lipophilicity than the non-prenylated precursor. However, the discrepancy between their enhanced lipophilicity and limited bioavailability in experimental findings highlighted the importance on understanding their permeability, absorption and metabolism through the *in vitro* Caco-2 cell permeability model. However, their high affinity to biological membranes, which leads to the higher basal cytotoxic potency and the lower recovery efficiency from cells, posed challenges on the Caco-2 cell permeability assay. To deal with these problems, this study investigated the nominal concentration range of wighteone and 6-prenlynaringenin used in Caco-2 cell permeability assay by measuring their basal cytotoxicity and the highest non-toxic concentration towards proliferating Caco-2 cells. In addition, the recovery factors of wighteone extracted from medium and cells by methanol and acetonitrile with 0.1% (*v/v*) formic acid was analysed and compared to optimize the extraction procedure for future Caco-2 cell permeability assay. The results showed that the basal cytotoxic potency of wighteone was higher than 6-prenlynaringenin. However, the nominal concentration of wighteone and 6-prenylnaringenin used in Caco-2 cell permeability model was still unclear, due to the unknow relationship between their basal cytotoxicity towards proliferating and differentiated Caco-2 cell. The extraction efficiency of methanol and acetonitrile with 0.1% (*v/v*) formic acid were both lower than 90%, and it showed no significant different between their recovery factors.
Keywords: Prenylated phenolics; Caco-2 cells; lipophilicity; basal cytotoxicity; recovery efficiency

1. Introduction

Abbreviations

WIG	wighteone	6-PN	6-prenylnaringenin
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry	PBS	Phosphate-Buffered Saline
		SDS	Sodium dodecyl sulfate
LDH assay	Lactate dehydrogenase assay	MeOH	Methanol
CFDA-AM assay	5-carboxyfluorescein diacetate acetoxymethyl ester assay	TM	Transport medium
		MEM	Minimum Essential Medium
BCA assay	Bicinchoninic Acid assay	SARs	Structure-activity relationships
IC_{10} or IC_{50}	Inhibitory concentration 10% or 50%	DMSO	Dimethyl sulfoxide
EC_{10} or EC_{50}	Effective concentration 10% or 50%	BSA	Bovine serum albumin stock
ACN with 0.1% (*v/v*) FA	Acetonitrile with 0.1% (*v/v*) formic acid		

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1.1. Prenylated phenolics showed potential benefits on human health

Prenylated phenolics are natural compounds present in various plants, especially in the roots, leaves and seeds of plants in the *Moraceace*, *Leguminosae* and *Asteraceae* family (Terao & Mukai, 2014). They were distinguished by the presence of a prenylated side chain attached to their flavonoid skeleton, which enhanced their bioactivity compared to non-prenylated precursors, making them promising lead compounds for the development of novel drugs (Osorio et al., 2021).

Prenylated phenolics had gained interest for their potential benefits to human health (Wang et al., 2001). Dietary intake of these so-called phytoestrogens as supplement may have a positive impact on treating menopausal complaints, hormone-related cancers, and osteoporosis (Simons et al., 2012). Furthermore, due to their strong antibacterial activity, prenylated phenolics could be used to treat human pathogenic fungi and microbial infections. Previous research indicated that prenylated phenolics could protect plants against diseases by significantly inhibiting bacterial and fungal activities (Chen et al., 2014). They were also known for their activity against methicillin-resistant Staphylococcus aureus (Ika Irianti et al., 2023) (Aelenei et al., 2020). In addition, past study demonstrated that some prenylated phenolics exhibit antitumor activity by inducting cell apoptosis and inhibiting angiogenesis across various cancers, including colorectal cancer, breast cancer, glioblastoma, and pancreatic cancer (Ambrož et al., 2019). It means that prenylated phenolics were also likely to promise as anti-cancer compounds in future cancer treatment.

1.2. Structural characteristics

Prenylated phenolics are a class of naturally occurring compounds with structural diversity which was characterized by the presence of a prenylated side chain (prenyl, geranyl) in the flavonoid's skeleton (Wang et al., 2001). Two subclasses included in flavonoids are shown in **Figure 1,** which are isoflavones and flavanones (de Souza Farias et al., 2021). Their chemical structures are different with the position of the B ring and the saturation between C2-C3.

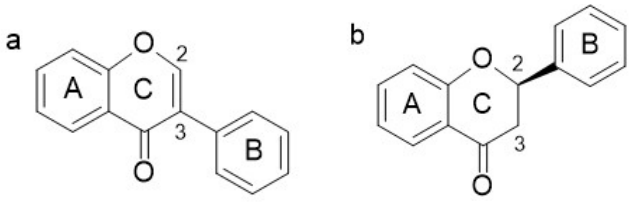
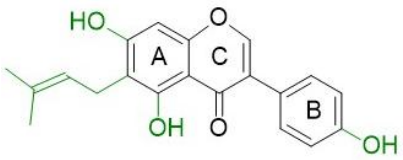
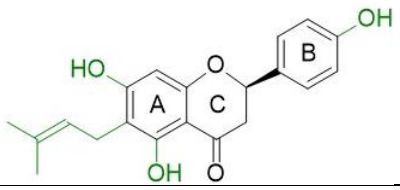


Figure 1. The chemical structure of (a) Isoflavones and (b) Flavanones. Drew by ChemDraw 23.1.2.

The structure and chemical properties of the compounds which were used in this study is shown in the **Table 1** below. Both WIG and 6-PN are included in the prenylated phenolics group. According to their chemical structure illustrated in **Table 1**, WIG and 6-PN is prenylated isoflavones and prenylated flavanones respectively.

Table 1. The structure (drew by ChemDraw 23.1.2.) and chemical properties of the prenylated phenolics used in this study

Name	Structure	Molecular Weight (g/mol)	Log P *	Log S **
Wightone (WIG)		338.4	3.86	-4.45
6-prenylnaringenin (6-PN)		340.4	3.17	-4.35

* Log P is partition coefficient which indicates the measure of the lipophilicity or hydrophobicity of a chemical compound. Calculated by ALOGPS 2.1 (Virtual Computational Chemistry Laboratory VCCLAB) Non-Java Interface.

**Log S represents the logarithm of a compound's solubility in water. Calculated by ALOGPS 2.1 (Virtual Computational Chemistry Laboratory VCCLAB) Non-Java Interface.

1.3. Structure-activity relationships

Understanding structure-activity relationships (SARs) helps relate biological activities, like cytotoxicity, to chemical structure, which would improve their potency and ensure sufficient bioavailability in future study. The lipophilicity of prenylated phenolics was concerned in previous research. The research has shown that the substitution of the flavonoid ring system with prenylated groups had the positive relationship with the increasing lipophilicity of prenylated phenolics as well as their higher affinity to biological membranes (Boozari et al., 2019). It would induce they showed higher bioactivity (Osorio et al., 2021), like basal cytotoxicity, by leading to an increased intracellular activity with higher cellular uptake and lower efflux (Lněničková et al., 2020).

The discrepancy between the high cellular uptake of prenylated phenolics, attributed to their enhanced lipophilicity, and the low bioavailability according to experimental findings (Cattoor et al., 2010) (Konishi et al., 2005) highlighted the critical role of efflux mechanisms in intestinal cells in the determination of bioavailability. Terao's (Terao & Mukai, 2014) study showed that the decreasing efflux from the cells would also result in that the presence of a prenyl side chain diminished the binding affinity of phenolics conjugating with adenosine triphosphate-binding cassette transporters. The low affinity of prenylated phenolics with transporters may lead to the slower excretion outside the tissue-constituting cells and be accumulated at a higher level in tissue (Boozari et al., 2019) (Terao & Mukai, 2014).

1.4. Caco-2 cell permeability assay and its potential problems

The metabolism and absorption of prenylated phenolics plays a key role on evaluating their bioavailability. The Caco-2 cells could be used as a human intestinal model to predict the intestinal permeability and absorption of drugs (Hubatsch et al., 2007). After Caco-2 cells differentiating to form tight junctions between cells, it could be served as a model of paracellular movement of compounds across the monolayer. In addition, Caco-2 cells express transporter proteins, efflux proteins, and Phase II conjugation enzymes to model a variety of transcellular pathways for test chemicals (van Breemen & Li, 2005).

However, the higher basal cytotoxicity of prenylated phenolics, increasing the rate of reducing cell viability and potential inducing apoptosis in Caco-2 cells. It would make an adverse impact on the prediction of intestinal permeability and absorption in Caco-2 cell permeability assay. In addition, the extraction efficiency of prenylated phenolics would also affect the outcome of Caco-2 cell permeability assay. To predict the absorption and permeability of prenylated phenolics, understanding their distribution in each part, such as the medium in apical and basolateral chamber, Caco-2 cell lines, and plastic well and inserts, would be crucial. However, due to the higher affinity to cell membrane of prenylated phenolics, their extraction efficiency from cells may be lower. Although the previous research provided some information about extraction solvent and methods to extract prenylated phenolics from plants, methanol revealed to be the most efficient solvent for extraction with recovery factors (%) which was more than 90% (Magalhães et al., 2007), research related to the solvent and procedure of extracting them from various and complex matrix, such as medium and Caco-2 cells, was still limited.

1.5. The aim of the thesis research

To deal with the present problems in Caco-2 cell permeability assay, two aims are supposed to be achieved in this thesis study. Firstly, assessing the basal cytotoxicity of WIG and 6-PN towards proliferating Caco-2 cells after 24 h and 48 h exposure would determine their nominal concentration range without compromising Caco-2 cell function in the permeability assay. Their basal cytotoxicity was measured in Lactate dehydrogenase (LDH) assay, Alamar Blue assay, 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) assay and Bicinchoninic Acid (BAC) assay and their inhibitory concentration 10% (IC_{10}), inhibitory concentration 50% (IC_{50}), effective concentration 10% (EC_{10}) and effective concentration 50% (EC_{50}) were also obtained in each assay. According to the Log P value of WIG and 6-PN shown in **Table 1**, WIG would illustrate more lipophilicity than 6-PN, due to its higher Log P value. It means that WIG is likely to shown higher basal cytotoxic potency than 6-PN, and lower concentrations without inhibiting effect on Caco-2 cells.

Additionally, to optimize the extraction efficiency of prenylated phenolics from medium and cells, its recovery was quantified using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) after extraction by methanol (MeOH) and acetonitrile with 0.1% (*v/v*) formic acid (ACN with 0.1% (*v/v*) FA). In study of Nikolić D (Nikolić & van Breemen, 2013) and Magalhaes P J (Magalhães et al., 2007), the recovery factor for extracting prenylated phenolics from hops by methanol was more than 90%. The extraction efficiency of WIG by MeOH might be higher than using ACN with 0.1% (*v/v*) FA. Meanwhile, the recovery factor of WIG extracted by MeOH from medium and Caco-2 cells may be more than 90%.

2. Materials and methods

2.1. Chemicals and reagents

The prenylated phenolics used in this study were WIG and 6-PN in Dimethyl sulfoxide (DMSO), which are shown in **Table 2** below. The culture medium contained Minimum Essential Medium (MEM) with 20% (*v/v*) fetal bovine serum, and 1% (*v/v*) penicillin/streptomycin/glutamine solution. The transport medium (TM) is MEM without phenol red. All the reagents used in this study are shown in **Table 3, 4** below.

Table 2. The purity and producer of the prenylated phenolics used in this study

Compound	Purity	Company
WIG	96%	MedChem Express (USA)
6-PN	94%	ChemFaces (China)

*The purity of WIG and 6-PN were measured in reversed-phase ultra-high performance liquid chromatography with photodiode array detection under UV280.

Table 3. The name, storage condition and producer of reagents used in this study.

Name	Storage condition	Producer/Company
Minimum Essential Medium (MEM)	4 ℃	Gibco, Thermo-Fisher Scientific (USA)
Phosphate-Buffered Saline (PBS) pH 7.4	4 ℃	Gibco™, Thermo-Fisher Scientific (UK)
Sodium dodecyl sulfate (SDS)	RT*	Merck, Sigma-Aldrich® Solution (USA)
Bovine serum albumin stock (BSA)	RT	Thermo-Fisher Scientific (USA)
BCA Protein assay working reagent A&B	RT	Thermo-Fisher Scientific (USA)
Alamar Blue™ HS Cell Viability Reagent	4℃	Invitrogen, Thermo Fisher Scientific (USA)
5-Carboxyfluorescein diacetate, acetoxymethyl ester (5-CFDA, AM)	4 ℃	Invitrogen, Thermo Fisher Scientific (USA)
HPLC-grade methanol (MeOH)	RT	Biosolve Chimie (France)
HPLC-grade acetonitrile (ACN)	RT	Biosolve Chimie (France)
Formic acid (FA)	RT	VWR® Chemicals (France)
Dimethyl sulfoxide (DMSO)	RT	Supelco, EMSURE® (Germany)

* RT means room temperature

Table 4. The name, preparation, storage condition and producer of reagents in TOX general stock.

Name	Preparation of Aliquots	Storage	Producer/Company
Fetal bovine serum	50 mL aliquot in the falcon tubes (50 mL)	-20 ℃	Gibco, Thermo-Fisher Scientific (USA)
Penicillin/streptomycin/glutamine solution	5mL aliquot in the falcon tubes (5 mL)	-20 ℃	Gibco™, Thermo-Fisher Scientific (USA)
Trypsin	Diluted 10 times in PBS. 50mL aliquot in the falcon tubes (50 mL)	-20 ℃	Gibco, Thermo-Fisher Scientific (Canada)
Lithium L-lactate	980 mg in 50 mL H_2O 5 ml aliquots in 15 mL Falcon tubes	-20 ℃	Merck, Sigma-Aldrich® Solution (USA)
B-Nicotinamide adenine dinucleotide sodium salt	172 mg in 46 mL H_2O 4.6 mL Aliquots in 15 ml Falcon tubes	-20 ℃	Merck, Sigma-Aldrich® Solution (USA)
Iodonitrotetrazolium chloride	66 mg in 2 mL DMSO 200 µl aliquots in 1.5 mL amber Eppendorf tubes (light sensitive)	-20 ℃	Merck, Sigma-Aldrich® Solution (USA)
Phenazine methosulfate	18 mg in 2 mL H_2O 200 µl aliquots in 1.5 mL amber Eppendorf tubes (light sensitive)	-20 °℃	Merck, Sigma-Aldrich® Solution (USA)
TRIS Buffer	Add 22.2 mg of Tris-HCl and 10.6 mg of Tris-base to 1 L water	RT	Merck, Sigma-Aldrich® Solution (USA)
1M H_2SO_4	Slowly adding 27,8 mL (of 18 M H_2SO_4 stock) to 473 mL H_2O	RT	--
MilliQ Water with 0.1% (*v/v*) FA	Add 5 mL formic acid to 5 L MilliQ water	RT	VWR® Chemicals (France)

Acetonitrile with 0.1% (*v/v*) FA	Add 5 mL formic acid to 5 L acetonitrile	RT	Biosolve Chimie (France) VWR® Chemicals (France)

2.2. Sample preparation and dilution

The prenylated phenolics were dissolved in DMSO at a concentration of 20 mM, which is referred to as the master stock solution. The master stock solution of each compound was diluted at least 200 times to make the dosing solution with less than 0.5% DMSO (*v/v*). The detailed dilution procedure is shown in *Appendix A.1*.

2.3. Caco-2 cell line culturing

Caco-2 cell line was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Caco-2 cells were cultured in MEM, in a humidified atmosphere of 5% CO_2 at 37 °C until 80–90% confluency. The cells were passaged twice per week to avoid differentiation caused by confluency >90%. Dead cells were washed out by removing the culture medium in the flasks and adding 10 mL PBS to wash out the remaining dead cells. The detachment was achieved by adding 3 mL trypsin and incubating under 37 ℃ for 5-10 minutes. The detachment reaction was stopped by adding 7 mL of culture medium into flasks. To avoid cell clustering, the cell flasks can be appropriately shaken to increase the contact of trypsin with the cells. The number of cells was measured by auto-counting (Cellometer® Auto T4 Plus Cell Counter, Nexcelom Bioscience LLC, USA). The volume of cell suspension was calculated by the planned cell numbers based on the passage time, which is 7.5 $\times 10^5$ cells/ml for four days passaging and 15 $\times 10^5$ cells/ml for three days passaging. The total volume of cell suspension and the culture medium was 10 mL. The Caco-2 cell line was cultured at 37 °C with 5% CO_2 in the incubator.

2.4. Optimization of extraction efficiency of WIG by using MeOH or ACN with 0.1% (v/v) FA

According to the log P value of WIG and 6-PN in **Table 1**, WIG showed higher lipophilicity than 6-PN. It means that it may have a lower recovery percentage from cells than 6-PN. To ompare the extraction efficiency between MeOH and ACN with 0.1% (*v/v*) FA and optimize the extraction procedure and solvent of prenylated phenolics for the future Caco-2 permeability assay, WIG was chosen to be used in this experiment. The test groups were two groups of Caco-2 cell suspension with 50 μM WIG and after one hour exposure, the WIG in medium and cells were extracted by MeOH and ACN with 0.1% (*v/v*) FA respectively. The control group was the transport medium with 50 μM WIG and incubated with test groups. And then WIG in medium and cells were extracted by MeOH. Each group included technical triplicates.

Preparing 2 mL transport medium and Caco-2 cell suspension (with 1.0 × 10^6 cells/mL) in glass vials (4 mL screw vial), add 10 μL/vial 10 mM WIG in DMSO in each vial and aliquoted 200 μL/vial in glass vials (1.5 mL short threat vials with 250 μL glass insert). After the one-hour incubation under 37 ℃ with 5% CO_2 in the incubator, all glass vials were centrifuged at 8524 g for 5 minutes until the pellet could be observed clearly in the bottom of glass insert. Then 30 μL supernatant was added into 60 μL MeOH or ACN with 0.1% (*v/v*) FA for extracting WIG from medium. Followed by washing step, the pellet was washed by the transport medium after removing all the supernatant out from the glass insert. After adding 200 μL MeOH or ACN with 0.1% (*v/v*) FA into glass vials, all glass vials were kept under room temperature for 10 minutes and then frozen at -20°C to release the WIG from cells to the solvent. After thawing, they were centrifuge at 8524 g for 10 minutes, and 60 μL supernatant was added into 30 μL TM. All the extraction samples were stored at -20 °C. Before the Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) measuring, all the samples were centrifuged at 8524 g for 5 minutes and diluted 10 times by combining 50 μL supernatant with 450uL MeOH: TM=2:1 (*v/v*) for LC-MS measuring.

2.5. Cytotoxicity assays for measuring the basal cytotoxic potency of WIG and 6-PN

The cytotoxicity of WIG and 6-PN was measured by the following four assays in 96-well plate, which are LDH assay, Alamar Blue assay, CFDA-AM assay and BAC assay. As shown in **Figure 2**, the wells in outline (green part) contained 200 μL culture medium without Caco-2 cells, and other wells (except the green part) contained 200 μL cell suspensions with 1.0× 10^5 cells/mL. In each biological replicate, two plates were exposed for 24 h and 48 h respectively. Each concentration of WIG and 6-PN, ranging from 0.78 μM to 100 μM, contained technical triplicates.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Medium	TM	0.5% DMSO in TM	WIG 0.78 µM	WIG 1.56 µM	WIG 3.125 µM	WIG 6.25 µM	WIG 12.5 µM	WIG 25 µM	WIG 50 µM	WIG 100 µM	Medium
B	Medium	TM	0.5% DMSO in TM	WIG 0.78 µM	WIG 1.56 µM	WIG 3.125 µM	WIG 6.25 µM	WIG 12.5 µM	WIG 25 µM	WIG 50 µM	WIG 100 µM	Medium
C	Medium	TM	0.5% DMSO in TM	WIG 0.78 µM	WIG 1.56 µM	WIG 3.125 µM	WIG 6.25 µM	WIG 12.5 µM	WIG 25 µM	WIG 50 µM	WIG 100 µM	Medium
D	Medium	TM	0.5% DMSO in TM	WIG 0.78 µM	WIG 1.56 µM	WIG 3.125 µM	WIG 6.25 µM	WIG 12.5 µM	WIG 25 µM	WIG 50 µM	WIG 100 µM	Medium
E	Medium	SDS 7.81µM	SDS 62.5 µM	6-PN 0.78 µM	6-PN 1.56 µM	6-PN 3.125 µM	6-PN 6.25 µM	6-PN 12.5 µM	6-PN 25 µM	6-PN 50 µM	6-PN 100 µM	Medium
F	Medium	SDS 15.63 µM	SDS 125 µM	6-PN 0.78 µM	6-PN 1.56 µM	6-PN 3.125 µM	6-PN 6.25 µM	6-PN 12.5 µM	6-PN 25 µM	6-PN 50 µM	6-PN 100 µM	Medium
G	Medium	SDS 30.25 µM	SDS 250 µM	6-PN 0.78 µM	6-PN 1.56 µM	6-PN 3.125 µM	6-PN 6.25 µM	6-PN 12.5 µM	6-PN 25 µM	6-PN 50 µM	6-PN 100 µM	Medium
H	Medium	TM	0.5% DMSO in TM	6-PN 0.78 µM	6-PN 1.56 µM	6-PN 3.125 µM	6-PN 6.25 µM	6-PN 12.5 µM	6-PN 25 µM	6-PN 50 µM	6-PN 100 µM	Medium

Figure 2. The layout of 96-well plate before being exposed to wighteone (WIG) and 6- prenylnaringenin (6-PN). TM means transport medium, and SDS means Sodium dodecyl sulfate. The yellow part was medium control. The grey part was vehicle control. The red part was positive control.

2.5.1 LDH assay

Cytotoxicity induced by WIG and 6-PN could be assessed by lactate dehydrogenase (LDH) leakage into the culture medium (Fotakis & Timbrell, 2006). The reaction mixes included 5 mL TRIS buffer, 5 mL Lithium L-lactate, 4.6 mL B-Nicotinamide adenine dinucleotide sodium salt, 200 µL Iodonitrotetrazolium chloride, and 200 µL Phenazine methosulfate. The 150 µL reaction mixes was added into each well in a new 96-well plate for further measuring, followed by mixing with 50 µL medium from the 96-well plate with Caco-2 cell lines. Because the reaction time in LDH assay was varied, the reaction was stopped by adding 100 µL 1M H_2SO_4 until the strong red colour was observed in the highest concentration of positive control. The absorbance of each well was measured under 490nm and 680nm in spectrophotometer (Infinite 200 Pro multimode microplate reader, TECAN Nanoquant, USA). The plastic impurities were removed by subtracting the absorbance under 680 nm from the absorbance under 490 nm. The percentage of cytotoxicity in each well was calculated by **eq. (1)** below (A means absorbance):

$$Cytotoxicity\ (\%) = \frac{A_{sample} - A_{TM}}{A_{SDS\ 250\mu M} - A_{TM}} \qquad \textbf{eq. (1)}$$

2.5.2 Alamar Blue & CFDA-AM assay

Alamar Blue and CFDA-AM assays were used to assess cell viability by measuring the fluorescence generated from the conversion of non-fluorescent substrates into fluorescent compounds through cellular metabolic activity in living cells (Kamiloglu et al., 2020). A mixture of these two dyes was prepared in TM prior to application to the cells such that the final dye concentrations were 5% (*v/v*) Alamar Blue and 15pM CFDA-AM. After removing all the medium in the plate with Caco-2 cell lines, the cells were washed by using TM (37 °C) for one minute. An aliquot of 100 µL of the working reagent was added to each well. After 1 h incubation in dark at 37 ℃ (with 5% CO_2), fluorescence in Alamar Blue assay (excitation 540 nm, emission 590 nm) and in CFDA-AM assay (excitation 493 nm, emission 541 nm) was measured in spectrophotometer (Infinite 200 Pro multimode microplate reader, TECAN Nanoquant, USA). The well exposed to same concentration of WIG or 6-PN but not containing Caco-2 cells was considered as the blank. Subtracting the fluorescence of blank from the fluorescence of sample exposed to same concentration of WIG or 6-PN, could get rid of the interfering of the colour of chemicals and medium. To calculate the cell viability the following **ep. (2)** was used (F means Fluorescence):

$$Cell\ viability\ (\%) = \frac{F_{sample} - F_{blank}}{F_{TM} - F_{blank}} \qquad \textbf{ep. (2)}$$

2.5.3 BCA assay

The BCA assay is a biochemical assay for the determination of total protein concentration. The increasing basal cytotoxic potency of WIG and 6-PN may reduce the number of viable cells or decrease cellular protein synthesis, both contributing to the decreasing protein concentration measured by the BCA assay (He, 2011). BCA assay was conducted after the removal of the working regent of Alamar Blue and CFDA-AM assay. The layout of the plate for BCA assay is shown in **Figure 3** below. Each well was washed by 100 µL PBS (37 °C) and the 25 µL MilliQ water was added to wells with cells. The wells in purple were added BSA standards. The plate was kept under -80 °C for 1 h to lyse the Caco-2 cells. After thawing the plate at room temperature, the MilliQ water was mixed properly with 200 µL BCA working regent (20 mL reagent A and 400 µL reagent B). And then the plate was incubated for 30 minutes in dark at 37 °C with 5% CO_2 in the incubator. The absorbance at 562nm was measured in spectrophotometer (Infinite 200 Pro multimode microplate reader, TECAN Nanoquant, USA). The absorbance

of the BSA standard was used to make a calibration curve which was used to determine the concentration of protein (μg/mL) in each well.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BSA 100 μg/mL	TM	0.5% DMSO in TM	WIG 0.78 μM	WIG 1.56 μM	WIG 3.125 μM	WIG 6.25 μM	WIG 12.5 μM	WIG 25 μM	WIG 50 μM	WIG 100 μM	BSA 100 μg/mL
B	BSA 50 μg/mL	TM	0.5% DMSO in TM	WIG 0.78 μM	WIG 1.56 μM	WIG 3.125 μM	WIG 6.25 μM	WIG 12.5 μM	WIG 25 μM	WIG 50 μM	WIG 100 μM	BSA 50 μg/mL
C	BSA 25 μg/mL	TM	0.5% DMSO in TM	WIG 0.78 μM	WIG 1.56 μM	WIG 3.125 μM	WIG 6.25 μM	WIG 12.5 μM	WIG 25 μM	WIG 50 μM	WIG 100 μM	BSA 25 μg/mL
D	BSA 12.5 μg/mL	TM	0.5% DMSO in TM	WIG 0.78 μM	WIG 1.56 μM	WIG 3.125 μM	WIG 6.25 μM	WIG 12.5 μM	WIG 25 μM	WIG 50 μM	WIG 100 μM	BSA 12.5 μg/mL
E	BSA 6.25 μg/mL	SDS 7.81μM	SDS 62.5 μM	6-PN 0.78 μM	6-PN 1.56 μM	6-PN 3.125 μM	6-PN 6.25 μM	6-PN 12.5 μM	6-PN 25 μM	6-PN 50 μM	6-PN 100 μM	BSA 6.25 μg/mL
F	BSA 3.13 μg/mL	SDS 15.63 μM	SDS 125 μM	6-PN 0.78 μM	6-PN 1.56 μM	6-PN 3.125 μM	6-PN 6.25 μM	6-PN 12.5 μM	6-PN 25 μM	6-PN 50 μM	6-PN 100 μM	BSA 3.13 μg/mL
G	BSA 1.56 μg/mL	SDS 30.25 μM	SDS 250 μM	6-PN 0.78 μM	6-PN 1.56 μM	6-PN 3.125 μM	6-PN 6.25 μM	6-PN 12.5 μM	6-PN 25 μM	6-PN 50 μM	6-PN 100 μM	BSA 1.56 μg/mL
H	BSA 0 μg/mL	TM	0.5% DMSO in TM	6-PN 0.78 μM	6-PN 1.56 μM	6-PN 3.125 μM	6-PN 6.25 μM	6-PN 12.5 μM	6-PN 25 μM	6-PN 50 μM	6-PN 100 μM	BSA 0 μg/mL

Figure 3. The layout of 96-well plate used in BCA assay. WIG means wighteone, and 6-PN means 6-prenylnaringenin. TM means transport medium, and SDS means Sodium dodecyl sulfate. BSA means Bovine serum albumin standard. The yellow part was medium control. The grey part was vehicle control. The red part was positive control. The purple part was BSA standards.

2.6. LC-MS/MS measuring

The LC-MS/MS used was a Shimadzu liquid chromatographic system (CBM-40lite) produced in Japan, coupled to a pump in low pressure gradient mode (LC-40D XR). The acquisition mode was Multiple Reaction Monitoring. Acquisition was performed with the software (LabSolutions LCMS). Eluent A was MilliQ Water with 0.1% (*v/v*) FA and Eluent B was ACN with 0.1% (*v/v*) FA, whose preparation is shown in **Table 4**. The column was a Kinetex C18 1.7 μm × 100 Å × 50 mm × 2.1 mm (Phenomenex 00B-4475 AN) non-polar column. The guard Column Holder (Phenomenex AJ0-9000) and guard Column (Phenomenex AJ0-8782) were made of the same material as the column. The injection volume was 1 μL. The flow rate was 312 μL/min. The interface was the electrospray ionization (ESI) was set to the negative ion mode and operated at 350 °C. The column oven temperature was set to 45 °C, and the autosampler temperature was maintained at 20 °C. The run time for each sample was 21 minutes. The peak with an *m/z* value of 337 in negative ion mode and a retention time of approximately 10.9 minutes was identified as WIG.

2.7. Data analysis

Data collected by LC-MS/MS was analysed in Lab Solution. The peak area was integrated in the clear and manual integration mode. The quantification was conducted by comparing peak area to each WIG standard in calibration curves. The WIG concentration recovered in control group was set as 100%. The unpaired t-test was performed in GraphPad (Prima 10) between the total extraction efficiency of WIG by MeOH and ACN with 0.1% (*v/v*) FA. It was also conducted on comparing the recovery factor of WIG from medium and cell separately by MeOH and ACN with 0.1% (*v/v*) FA.

In addition, the result of cytotoxicity assay was presented as the percentage of LDH released (% control) in LDH assay, the cell viability (% control) in Alamar Blue & CFDA-AM assay and protein concentration (μg/mL) in BCA assay. The dose-response curve for each assay after 24 h or 48 h exposure was fit by GraphPad (Prima 10) in Nonlinear Regression mode in XY analysis. The dose-response curve between the wig and 6-PN 's concentration (μM) and the LDH released (% control) was fit in stimulation equation, which was log (agonist) vs. response – Variable slope (four parameters) specifically. For rest three assay, their dose- response curve was fit in inhibition mode that was log (agonist) vs. response – Variable slope (four parameters). Meanwhile, the EC_{50} (in LDH assay) or IC_{50} (in Alamar Blue, CFDA-AM and BCA assay) were calculated according to dose-response curve by GraphPad (Prima 10). Their EC_{10} (set F value as 10) or IC_{10} (set F value as 90) were obtained in the dose-response curve fit in mode that [Agonist] vs. response – Find Ecanything (F value represents the percentage of the way between Bottom and Top).

Results were expressed as the mean ± standard deviation (SD) for the experiments with technical triplicates. If it contained two biological replicates, outcomes would be shown as the mean ± SD based on six replicates (technical triplicates in each biological replicate). In cytotoxicity assays, the mean ± SD values for technical triplicates were presented as mean value with error bar in the dose-response curve. The technical triplicates were expressed as single points in the bar chart of extracting WIG from medium and cells by MeOH and ACN with 0.1% (*v/v*) FA.

3. Results

3.1. In vitro cytotoxicity experiment of WIG and 6-PN in proliferating Caco-2 cell lines

This study evaluated the basal cytotoxicity of two prenylated phenolics, namely wighteone (WIG) and 6-prenylnaringenin (6-PN) in LDH assay, Alamar Blue assay, CFDA-AM assay and BCA assay and obtained their IC_{10} (inhibitory concentration 10%), IC_{50} (inhibitory concentration 50%), EC_{10} (effective concentration 10%) and EC_{50} (effective concentration 50%). It gave supports to determine the nominal concentration range that would not compromise Caco-2 cell function in the Caco-2 cell permeability assay.

The proliferating Caco-2 cells with 20 (first biological replicate) or 22 (second biological replicate) passages, were exposed to WIG and 6-PN within the concentration range from 0.78µM to 100 µM for 24 h and 48 h. Each concentration of WIG and 6-PN, medium control (TM) and vehicle control (TM with 0.5% (*v/v*) DMSO) contained technical triplicates. For each exposure duration, two biological replicates were conducted.

3.2.1 LDH assay

The cytotoxicity of WIG and 6-PN was determined by the amount of LDH in medium released by the cells. The dose-response curve between the concentration of WIG and 6-PN and the percentage of LDH released in the medium is shown in **Figure 4**.

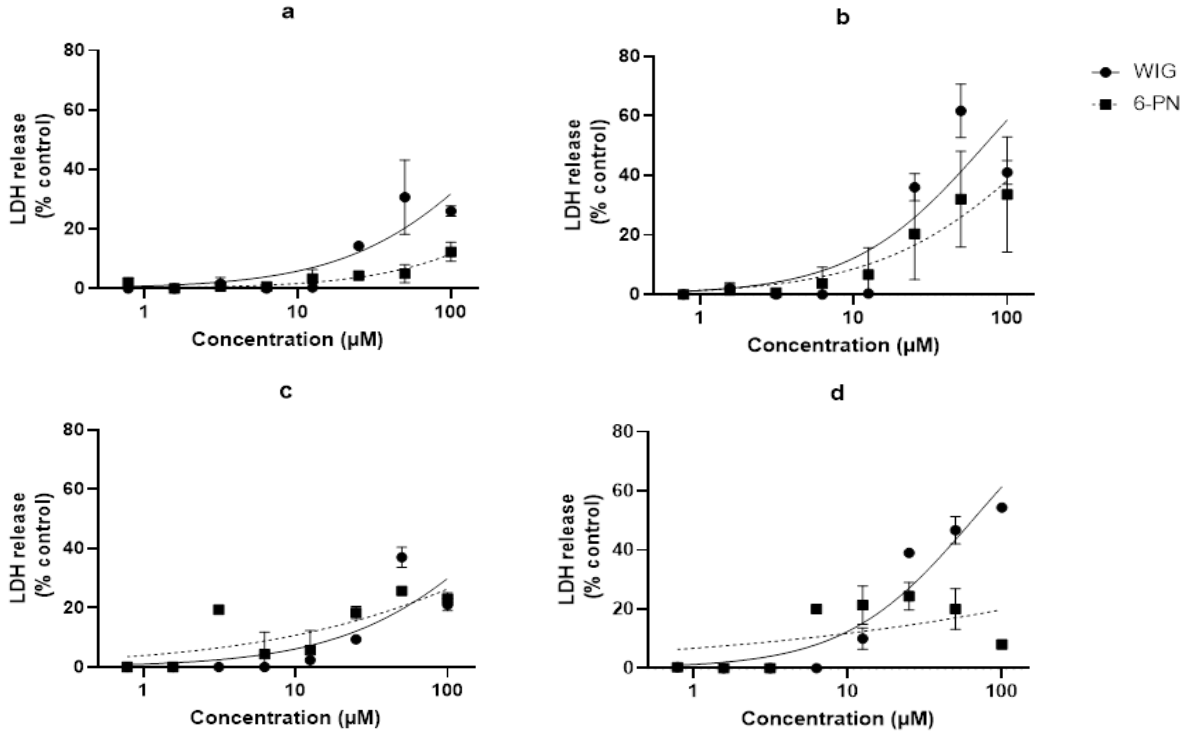


Figure 4. The effect of wighteone (WIG) and 6-prenylnaringenin (6-PN) on the percentage of LDH released in medium measured in LDH assay for proliferating Caco-2 cells (with 20 or 22 passages) after 24 h and 48 h treatment, with setting the absorbance of 250 µM Sodium dodecyl sulfate (SDS) as 100%. (a) and (b) is first biological replicate with 24 h and 48 h exposure time respectively. (c) and (d) is second biological replicate after 24 h and 48 h exposure respectively. The LDH release (% control) was expressed by mean and SD of technical triplicates.

As setting the absorbance of 250 µM SDS as 100% positive, both the LDH leakage after Caco-2 cells being exposed to 100 µM WIG and 6-PN did not reach 100%. The leakage of LDH after exposing under 6-PN was higher than the one exposure under WIG in low concentration range. Conversely, it showed a higher percentage of LDH release by exposing Caco-2 cells to high concentration WIG than 6-PN with same concentration. It means that the loss of cell viability after exposure under 3.125 µM to 12.5 µM 6-PN was more than WIG in same concentration. Meanwhile, WIG was more cytotoxic potent than 6-PN in higher concentration range between 25 µM and 100 µM.

3.2.2 Alamar Blue & CFDA-AM assay

The Almar Blue and CFDA-AM were non-fluorescent compounds which could be transferred into the chemicals with fluorescence through cellular metabolic activity. The cell viability after exposure to WIG and 6-PN was determined by measuring the fluorescence inside the cells (CFDA-AM assay) or in the medium (Alamar Blue assay). The higher fluorescent signal indicated more cellular reaction inside the living cells, which reflected the higher cell viability in this well. The dose-response between the concentration of WIG and 6-PN and the proportion of Caco-2 cell viability after 24 h and 48 h exposure is shown in **Figure 5** (Alamar Blue assay) and **Figure 6** (CFDA-AM assay).

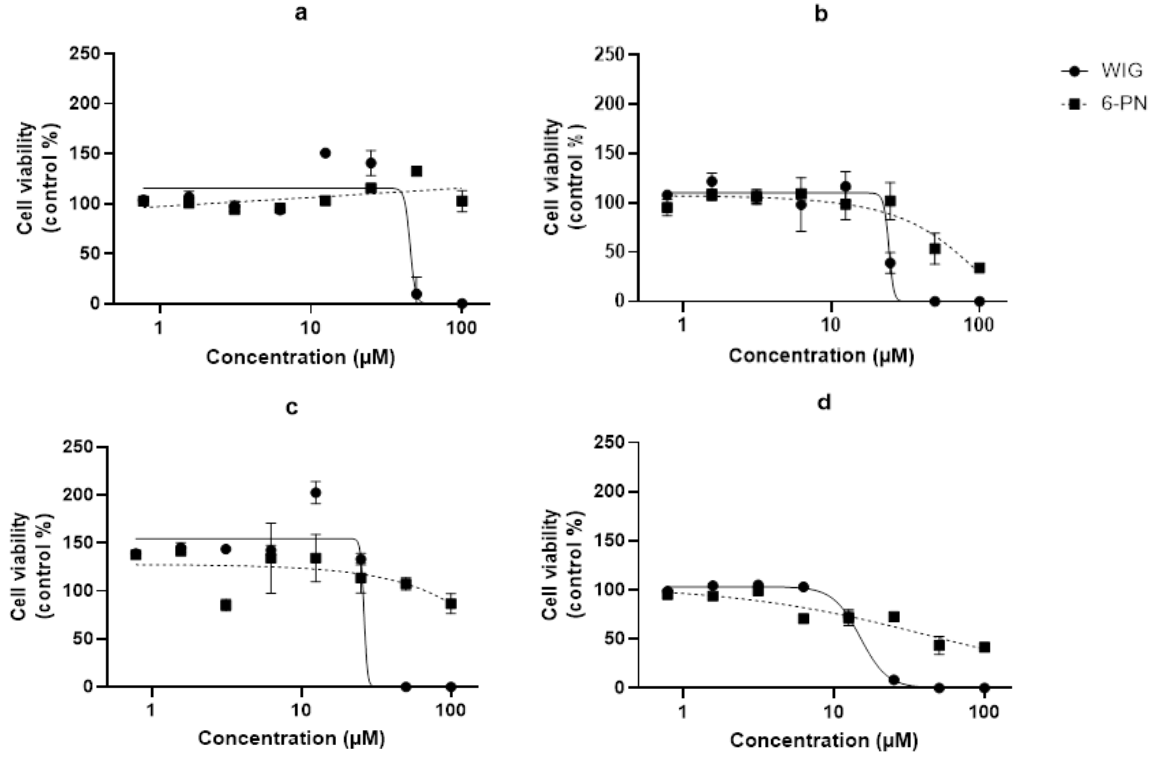


Figure 5. The effect of wighteone (WIG) and 6-prenylnaringenin (6-PN) on the percentage of cell viability in Alamar Blue assay for proliferating Caco-2 cells (with 20 or 22 passages) after 24 h and 48 h treatment, with setting the fluorescence of medium with 0.5% (*v/v*) DMSO as 100%. (a) and (b) is first biological replicate with 24 h and 48 h exposure time respectively. (c) and (d) is second biological replicate after 24 h and 48 h exposure respectively. Cell viability (control %) was expressed by mean and SD of technical triplicates.

In the Alamar Blue assay, 6-PN didn't show cytotoxic potency towards proliferating Caco-2 cells (with 20 or 22 passages) after 24 hours exposure, due to the percentage of cell viability after 24 h exposure to 100 µM 6-PN was 94.7% ± 11.8 (**Figure 5-a, c**). After being exposed to 100 µM 6-PN for 48 h, the cell viability (%) of Caco-2 cells decreased to 37.8% ± 4.1 (**Figure 5-b, d**). Besides, the percentage of cell viability was 0% after exposure to 100 µM WIG after Caco-2 cells exposed for both 24 h and 48 h (**Figure 5**). It reflected that long exposure duration would induce higher basal cytotoxicity. In addition, the cell viability (%) after being exposed to WIG was lower than 6-PN, which means that WIG showed higher cytotoxic potency on proliferating Caco-2 cells than 6-PN in high concentration ranging from 50 µM to 100 µM.

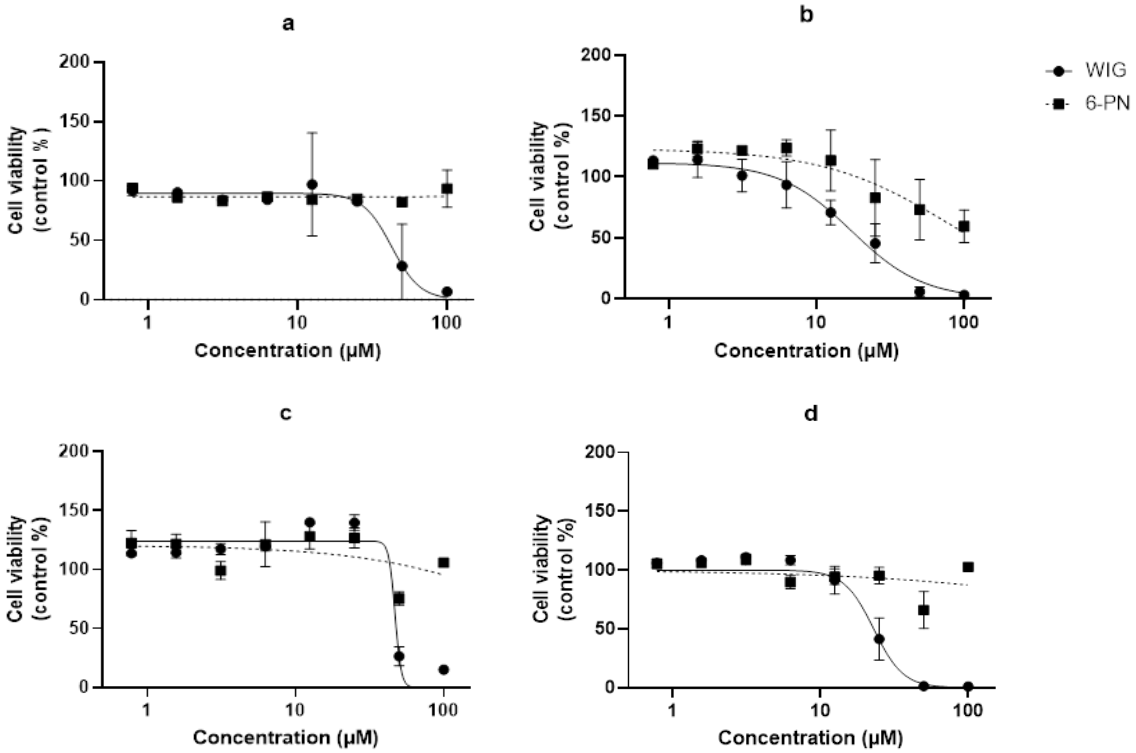


Figure 6. The effect of wighteone (WIG) and 6-prenylnaringenin (6-PN) on the percentage of cell viability in CFDA-AM assay for proliferating Caco-2 cells (with 20 or 22 passages) after 24 h and 48 h treatment, with setting the fluorescence of medium with 0.5% (*v/v*) DMSO as 100%. (a) and (b) is first biological replicate with 24 h and 48 h exposure time respectively. (c) and (d) is second biological replicate after 24 h and 48 h exposure respectively. Cell viability (control %) was expressed by mean and SD of technical triplicates.

The cell viability (%) of Caco-2 cells was 99.5% ± 11.6 and 81.0% ± 23.0 respectively after 24 h and 48 h treatment of 100 μM 6-PN (**Figure 6-a, c**). In the second biological replicate, cell viability (%) decreased with the increasing concentration of 6-PN from 50 μM to 100 μM (**Figure 6-c, d**). As shown in **Figure 6-a, c**, with concentration rising from 25 μM to 100 μM, the proportion of cell viability declined from 111.2% ± 28.8 to 10.8 % ± 4.2 after being exposed to WIG for 24 h. Meanwhile, the cell viability (%) was 3.5% ± 3.2 and 2.0% ± 2.4 respectively after being exposed by 50 μM and 100 μM WIG for 48 h (**Figure 6-b, d**), which was lower than the cell viability (%) after 24 h exposure. In CFDA-AM assay, Caco-2 cells showed lower cell viability after the treatment of high concentration of WIG than the 6-PN in the same concentration range, which determined that WIG indicated more cytotoxic potent than 6-PN in concentration range from 50 μM to 100 μM after 24 h exposure. Meanwhile, after 48 h exposure to WIG and 6-PN, the potency of 6-PN was lower than WIG in the range of concentration between 25 μM and 100 μM.

3.2.3 BCA assay

The BCA assay measured the total protein concentration in the cells. The dose-response relationship between the concentration (μM) of WIG and 6-PN and the concentration of protein (μg/mL) indicated the cell integrity, which means that a higher protein concentration reflected the higher cell viability indirectly. The dose-response curve in the BCA assay is illustrated in **Figure 7**.

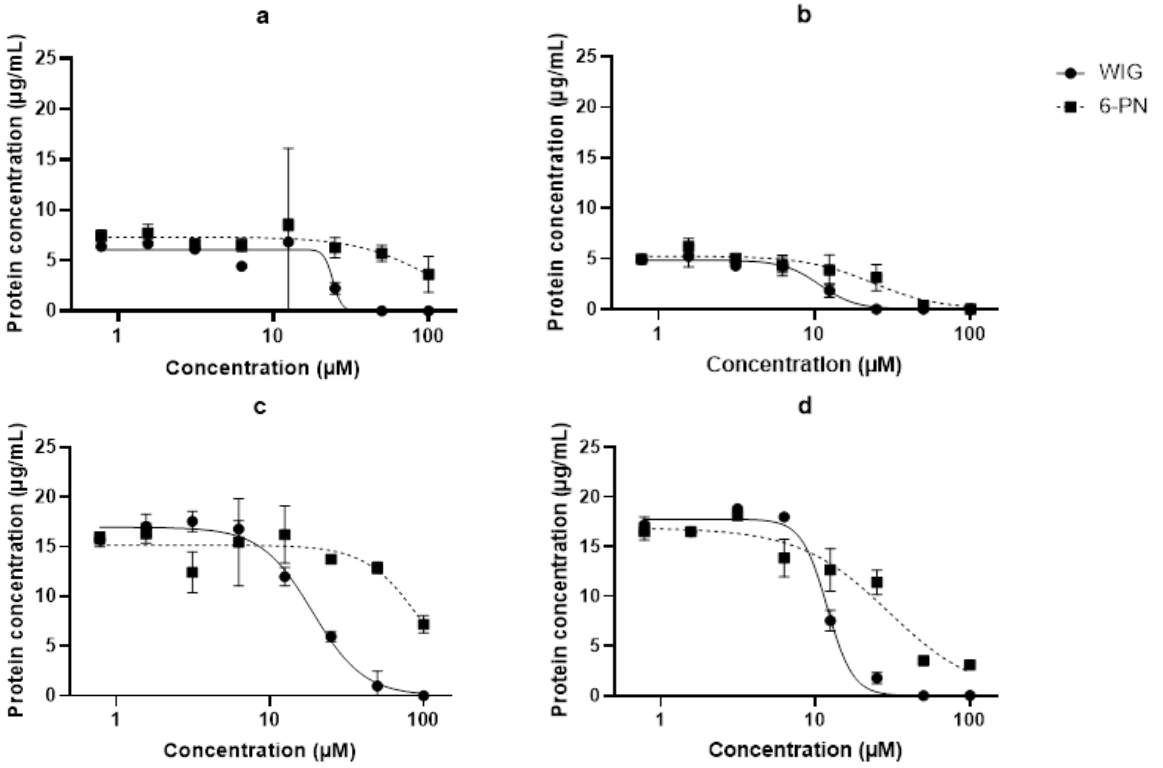


Figure 7. The effect of wighteone (WIG) and 6-prenylnaringenin (6-PN) on the total protein concentration in BCA assay for proliferating Caco-2 cells (with 20 or 22 passages) after 24 h and 48 h treatment. The total protein concentration was calculated by the calibration curve of BSA standard range from 0 µg/mL to 2000 µg/mL. (a) and (b) is first biological replicate with 24 h and 48 h exposure time respectively. (c) and (d) is second biological replicate after 24 h and 48 h exposure respectively. Protein concentration (µg/mL) was expressed by mean ± SD of technical triplicates.

Compared to the first biological replicate, the total protein concentration in second biological replicate was higher after the treatment of lowest concentration of WIG and 6-PN. As illustrated in **Figure 7a, 7c** and **7d**, the protein concentration in Caco-2 cells was lower after being exposed to high concentration range of WIG compared to same concentration of 6-PN, which means that WIG showed higher cytotoxic potent than 6-PN at concentration ranging from 12.5 µM to 100 µM. After comparing the protein concentration after being exposed to WIG and 6-PN after different exposure duration, the longer exposure time induced more cytotoxic potency for both WIG and 6-PN with concentration ranging from 12.5 µM to 100 µM.

3.2.4 The IC_{10}, IC_{50} and EC_{50} of WIG and 6-PN obtained from cytotoxicity assays

IC_{50} and EC_{50} are inhibitory concentration 50% and effective concentration 50% respectively. The EC_{50} was obtained in LDH assay to show as the concentration of WIG and 6-PN with 50% effects on LDH releasing, and the IC_{50} values determined from the Alamar Blue, CFDA-AM, and BCA assays represented the concentrations of WIG and 6-PN achieved a 50% inhibitory effect on the integrity of Caco-2 cells. The IC_{50} and EC_{50} value of WIG and 6-PN is illustrated in **Table 5**.

Table 5. The IC_{50} or EC_{50} of wighteone (WIG) and 6-prenylnaringenin (6-PN) in four cytotoxicity assays (LDH assay, Alamar Blue assay, CFDA-AM assay, BCA assay) after 24h and 48h exposure.

Compound		WIG		6-PN	
Exposure duration (h)		24	48	24	48
EC_{50} (μM)	LDH assay	>100	67.7 ± 3.3	>100	>100
IC_{50} (μM)	Alamar Blue assay	37.1 ± 9.1	19.7 ± 4.7	>100	53.8 ± 7.2
	CFDA-AM assay	43.1 ± 4.3	20.2 ± 2.9	Unstable	Unstable
	BCA assay	21.7 ± 2.7	11.5 ± 0.5	97.5 ± 0.9	26.7 ± 3.1

Due to the unstable signals in wells exposed by 6-PN in CFDA-AM assay (**Figure 5, 6**), there was lack of the IC_{50} value of 6-PN after 24 h and 48 h exposure in CFDA-AM assay. According to the existing EC_{50} or IC_{50} value, the value of EC_{50} and IC_{50} of both WIG and 6-PN after 48 hours exposure was lower than the value of being exposed for 24 h. It showed that the longer exposure duration would lead to a higher cytotoxic potency of WIG and 6-PN for proliferating Caco-2 cells (with 20 or 22 passages). The EC_{50} of WIG and 6-PN obtained in LDH assay was higher than the one obtained in other assays, especially the EC_{50} of 6-PN were both more than 100 μM after 24 h and 48 h exposure. In addition, comparing the IC_{50} of WIG in the rest of three assays, the IC_{50} obtained in CFDA-AM assay was highest, and lowest IC_{50} value was obtained in BCA assay.

The IC_{10} is inhibitory concentration 10%, which could be used to represent the concentration of WIG and 6-PN with low or without adverse effects on Caco-2 cells. The IC_{10} value of WIG and 6-PN is shown in **Table 6**.

Table 6. The IC_{10} or EC_{10} of wighteone (WIG) and 6-prenylnaringenin (6-PN) in four cytotoxicity assays (LDH assay, Alamar Blue assay, CFDA-AM assay, BCA assay) after 24h and 48h exposure.

Compound		WIG		6-PN	
Exposure duration (h)		24	48	24	48
EC_{10} (μM)	LDH assay	19.2 ± 0.0	7.9 ± 0.0	45.2 ± 36.7	8.6 ± 3.6
IC_{10} (μM)	Alamar Blue assay	33.8 ± 9.0	15.5 ± 6.0	>100	11.3 ± 9.7
	CFDA-AM assay	34.2 ± 8.0	9.4 ± 4.4	Unstable	Unstable
	BCA assay	14.5 ± 6.4	6.9 ± 0.9	34.4 ± 4.7	7.4 ± 0.6

No reliable IC_{10} value after Caco-2 cells being exposed to 6-PN was obtained in CFDA-AM assay, because dose-response curve didn't fit based on the irregular signal (**Figure 6**). In each cytotoxicity assay, the IC_{10} value would be lower after 48 h exposure compared to 24 h exposure. It means that a lower concentration of WIG and 6-PN that may induce the inhibiting effect on Caco-2 cells due to the longer exposure duration. Although the IC_{10} value of 6-PN was lower than WIG after 48 h exposure in Alamar Blue assay, based on the IC_{10} value in LDH and BCA assay in **Table 6**, the concentration of WIG without causing adverse effects on cells was lower than 6-PN with the same exposure time.

3.2 Optimization of extraction efficiency of WIG from medium and Caco-2 cells by using MeOH or ACN with 0.1% (v/v) FA

The higher lipophobicity of prenylated phenolics than their non-prenylated precursor led to a higher possibility to bind with cell membrane, which would have an adverse impact on their extraction efficiency from cells in future Caco-2 cells permeability assay. To optimize the extraction procedure and solvent of prenylated phenolics for the future Caco-2 permeability assay, Caco-2 cells were exposed to 50 μM WIG for 1 h and WIG was recovered from

medium and Caco-2 cells by methanol (MeOH) or acetonitrile (ACN) with 0.1% (*v/v*) formic acid (FA). The distribution of WIG recovered from the medium and cells in each group is illustrated in **Figure 8**.

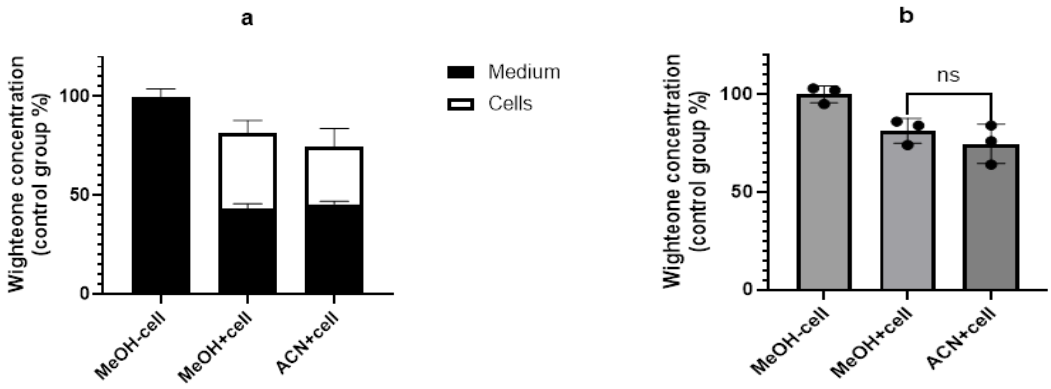


Figure 8. The concentration of WIG in test groups with cells extracted by MeOH or ACN with 0.1% (*v/v*) formic acid and the control group without cells extracted by MeOH. Each group had technical triplicates. The concentration of WIG found back in MeOH-cell group was set as 100%. (a) is the bar chart shown the concentration of WIG in each compartment. (b) contains the single points for each technical replicate and the unpaired t-test between two test groups.

The WIG extracted from cells by MeOH and ACN with FA showed recovery of WIG with 82% and 75% respectively, but no significant difference was shown between their recovery factor with T (3.4) = 0.97, P = 0.397. The recovery proportion of WIG extracted by MeOH and ACN with 0.1% (*v/v*) FA from medium was 43% and 45% respectively, which had no significant variance with T (3.2) = 1.31, P = 0.273. However, the extraction percentage of WIG from cells extracted by MeOH was 39%, which was higher than the percentage of extraction from cells by ACN with 0.1% (*v/v*) FA with value of 29%. However, it didn't show dramatic difference between them (T (3.6) = 1.40, P = 0.242).

4. Discussion

To determine the basal cytotoxicity potency and concentration of wighteone (WIG) and 6-prenylnaringenin (6-PN) without inducing adverse impact on Caco-2 cells, the Caco-2 cell line was exposed to WIG and 6-PN with concentration, ranging from 0.78 µM to 100 µM, for 24 hours and 48 hours. Their basal cytotoxicity was measured in LDH assay, Alamar Blue assay, CFDA-AM assay and BCA assay. The IC_{10} value of WIG and 6-PN obtained in these four assays were used to evaluate their highest non-toxic concentration towards proliferating Caco-2 cells, which would be used as the nominal concentration of WIG and 6-PN in Caco-2 permeability assay. In addition, quantifying and comparing the recovery efficiency of extracting WIG from medium and Caco-2 cells by using MeOH and ACN (containing 0.1% (*v/v*) FA), supported the optimization of extraction procedure of prenylated phenolics from medium and cells in further Caco-2 cell permeability assay.

4.1 The cytotoxicity of wighteone and 6-prenylnaringenin towards proliferating Caco-2 cells

After comparing the LDH released (% control) in LDH assay, cell viability (% control) of Caco-2 cells in Alamar Blue and CFDA-AM assay, and the concentration of protein in BCA assay after exposing Caco-2 cells to different concentration WIG and 6-PN, range from 0.78 µM to 100 µM, for 24 and 48 hours, WIG showed more cytotoxic potency than 6-PN in high concentration range between 25 µM to 100 µM. It fulfilled the hypothesis that WIG may show higher cytotoxicity than 6-PN, because of its higher lipophilicity, which would induce the increasing possibilities to bind with cell membrane and make effort on cells.

The EC_{50} obtained from LDH assay was much higher than the IC_{50} obtained from the rest of the assays, but the EC_{10} in LDH didn't show same trend among these four cytotoxicity assays. Its possible reason would be the low water solubility of WIG and 6-PN, which is shown in **Table 1**. It means that the freely available concentration in each well was not as same as the nominal concentration, especially in the high concentration range from 50 µM to 100 µM. It would lead to the situation that the percentage of LDH released wouldn't reach 100% which was set by the absorbance of LDH released after exposure to 250 µM Sodium dodecyl sulfate (SDS), with increasing concentration of WIG and 6-PN to 100 µM.

CFDA-AM and Alamar Blue are compounds used in fluorometric cell viability assays. Following the nonspecific enzymatic activity of esterases, CFDA-AM was converted into fluorescent substance and stayed inside the cellular membrane of living cells. As for Alamar Blue assay, Alamar Blue was reduced into resorufin in the enzymatic reaction inside cells, which would be extracted from the living cells into the medium and resulted in a change in the colour of the medium (Kamiloglu et al., 2020). It means that the overlapping cell line, as shown in **Figure 9**, may increase the fluorescence in the CFDA-AM assay due to the higher signal in the limited area, but would not make impact on the result in Alamar Blue assay. The overlapping cell line encountered after being exposed to 6-PN in concentration range from 3.125 µM to 100 µM. It could explain the reason why the fluorescent signal of some wells being exposed to 6-PN was higher than the fluorescent signal of vehicle control (medium with 0.5% (*v/v*) DMSO) which was set as 100%, especially the wells after 48 h exposure to 6-PN in first biological replicate and 24 h exposure to 6-PN in second biological replicate (**Figure 6-b, c**)

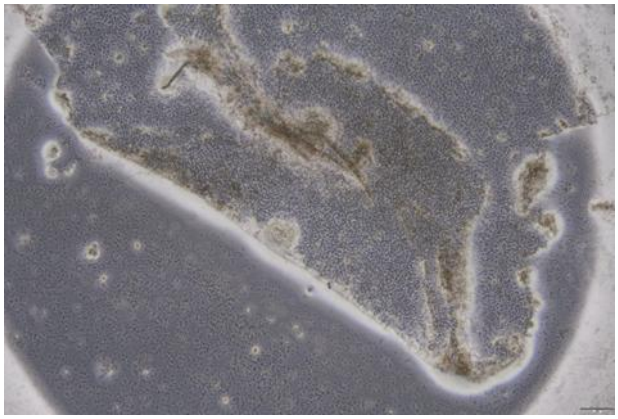


Figure 9. The microscope picture of Caco-2 cell line in the second technical replicate of 3.125 µM 6-prenylnaringenin (6-PN) with 24h exposure duration in the second biological replicate. This picture was taken when Caco-2 cell lines were washed by 37℃ PBS after measuring the fluorescence in Alamar Blue and CFDA-AM assay.

Table 7. The total concentration of protein in Caco-2 cell lines obtained in BCA assay in first and second biological after being exposed to 0.78 µM WIG and 0.78 µM 6-PN for 24 h and 48 h. Each total concentration of protein was presented by mean ± SD of technical triplicates.

Exposure duration (h)	First biological replicate		Second biological replicate	
	WIG	6-PN	WIG	6-PN
24	6.4 ± 0.2	7.4 ± 0.5	15.7 ± 0.6	15.9 ± 0.5
48	4.9 ± 0.4	4.9 ± 0.2	17.2 ± 0.6	16.5 ± 0.7

As shown in **Table 7**, the total concentration of protein in Caco-2 cells in first biological replicate after being exposed to 0.78 µM WIG and 6-PN was lower than the total protein concentration in Caco-2 cells in second biological replicate. The possible reason would be the changing of the experiment procedure. In the first biological replicate, 50 µL MilliQ water was added into each well in the plates with Caco-2 cell lines. Then, after the Caco-2 cells releasing the protein into MilliQ water after 1 hour freezing at -80 ℃, 25 µL MilliQ water was transferred into a new plate without cells. However, in second biological replicate, the 25 µL MilliQ water was added into the wells in the plates with Caco-2 cell lines directly. Compared to the second biological replicate, the concentration of total protein in the first biological replicate was diluted 2 times. However, the protein concentration measured in the second biological replicate was 2.5 to 3.2 times higher than the ones obtained in the first biological replicate. This discrepancy was likely attributed to the inhomogeneity of protein in the wells, possibly due to insufficient mixing caused by inadequate pipetting up and down prior to sample transferring, resulting in a lower protein concentration in the MilliQ water in the first biological replicate.

In addition, the IC_{50} and IC_{10} obtained in BCA assay was lower than the rest three assays, which indicated WIG and 6-PN showed higher cytotoxic potency and lower concentrations without adverse impact on Caco-2 cells. The previous studies showed that prenylated phenolics would interact at the target site (cell membrane and/or protein) to reveal the effect due to its elevation of hydrophobicity and lipophilicity (Arung et al., 2010) (Sun et al., 2015). It indicated that prenylated phenolics may interact with the enzyme and protein inside the Caco-2 cells, which would lead to the modification of protein structure. It was likely to induce the underestimation of protein concentration in a BCA assay, which showed a higher cytotoxic potency on WIG and 6-PN. This situation was more likely to encounter after being exposed to high concentration of WIG and 6-PN, because the increasing freely available concentration led to more reactions with protein. It may explain the reason why the IC_{10} and IC_{50} obtained in BCA assay was lowest in these four cytotoxicity assays.

4.2 The concentration of WIG and 6-PN with low or no adverse impact on proliferating Caco-2 cells after 24 h and 48 h exposure

According to the highest non-toxic concentration of WIG and 6-PN in **Table 6**, the highest non-toxic concentration of WIG and 6-PN towards proliferating Caco-2 cells were 25.4 µM ± 11.1 and 39.8 µM ± 26.7 respectively after 24 h exposure. As for after 48 hours exposure, the concentrations of WIG and 6-PN with low or no adverse impact on proliferating Caco-2 cells decrease to 9.9 µM ± 5.0 and 9.1 µM ± 6.2 respectively. WIG showed lower concentration with no or low inhibiting effect on Caco-2 cells than 6-PN. Meanwhile, extending exposure time from 24 h to 48 h would reduce the gap between the IC_{10} value of WIG and 6-PN.

The Caco-2 cell lines used in Caco-2 cell permeability assay are supposed to be differentiated by culturing in the plate for 21 days and the medium should be refreshed every 48 hours to make Caco-2 cells form tight junctions between cells. However, the Caco-2 cell lines used in this study were proliferating Caco-2 cells, which were cultured for 2 days in the 96-well plates. In Sarah's study (van Dinteren, 2024), after being exposed to Glabridin, Licochalcone and Glycycoumarin for 4 hours, the highest non-toxic concentrations measured in LDH assay and non-reducing cell viabilities measurd in water-soluble tetrazolium salt-1 (WST-1) assay between proliferating (cultured for 2 days) and differentiated (cultured for 21 days) Caco-2 cells were not significantly different. It means that the concentration range of WIG and 6-PN with no or low adverse impact on proliferating Caco-2 cells could be used as the nominal concentration range in Caco-2 cells permeability assessment. However, in another study from Ambrož (Ambrož et al., 2019), xanthohumol, isoxanthohumol, 6-PN, and 8-prenylnaringenin exhibited significantly greater basal cytotoxic effects on proliferating Caco-2 cells compared to differentiated Caco-2 cells. It indicated that the highest non-toxic concentration of WIG and 6-PN obtained from cytotoxicity assays by using proliferating Caco-2 cell lines would be lower than the one towards differentiated Caco-2 cells. Even though, according to the result from these two studies, the highest non-toxic concentration of WIG and 6-PN obtained in this study could be used as the nominal concentration in Caco-2 cells permeability assessment without inhibiting the viability and function of Caco-2 cell, further research about determining the basal cytotoxicity of WIG and 6-PN towards differentiated Caco-2 cells is needed.

4.3 Optimizing the efficiency of recovering WIG from medium and Caco-2 cells by MeOH and ACN with 0.1% (v/v) FA for Caco-2 cell permeability assay

The recovery of prenylated phenolics played a key role on predicting their permeability, absorption and metabolism in the Caco-2 cell permeability assay. Due to the higher lipophilicity of prenylated phenolics, the possibility of binding with cell membrane would increase. It posed challenges on their recovery efficiency in Caco-2 cell permeability assay. Although the studies related to extracting efficiency of recovering prenylated phenolics from plant by MeOH was more than 90% (Nikolić & van Breemen, 2013) (Magalhães et al., 2007), the research investigating the recovery factor of WIG extracted from medium and cells was still limited.

According to **Figure 8**, the recovery factor of WIG extracted from medium and cells by MeOH and ACN with 0.1% (*v/v*) FA were both lower than 90%, and they were not dramatic different. The possible reason would be its higher affinity to biological membranes decreased their extraction efficiency from cells. Meanwhile, the loss of cells was observed before the cell extraction. After taking sample for medium extraction, the medium in each glass vial was removed. Before extracting the WIG from Caco-2 cells, the pellet and the glass insert were washed by pure transport medium to wash out the medium left on pellet and in the glass insert. During this process, the cell pellet was touched and stuck on the pipette tips when pipetting the medium and transport medium out. It would cause the low recovery of WIG from cells in final analysis due to the loss of Caco-2 cells containing WIG before the cell extraction.

In addition, the calibration curve of WIG with concentration, ranging from 0.0412 µM to 10 µM, was measured before and after the measurement of samples. As shown in **Figure 12** in *Appendix A.2*, the peak area of WIG in the calibration curve was reduced in the second measurement (after the measurement of sample). It means that the peak area of samples would be lower than expected, due to the decreasing signal over time in the LC-MS/MS measurement. The lower peak area in the measurement of samples used in the quantification of WIG would cause the illusion of a lower extraction concentration of WIG.

4.4 Potential solutions and optimization for follow-up studies

In previous studies, they showed different outcomes about the basal cytotoxicity of prenylated phenolics towards proliferating and differentiated Caco-2 cells. It posed challenges on predicting the nominal concentration range in Caco-2 cell permeability assay based on the highest non-toxic concentration of WIG and 6-PN towards proliferating Caco-2 cells. To obtain the concentration of WIG and 6-PN without adverse impact for the future

Caco-2 cell permeability assay, it's necessary to measure their basal cytotoxic potency towards differentiated Caco-2 cells.

After analysis of the results obtained from LDH assay, Alamar Blue assay, CFDA-AM assay and BCA assay, the basal cytotoxicity of WIG and 6-PN was not suitable to be measured in LDH assay. The main reason was that their low water solubility induces a discrepancy between free available concentration and nominal concentration, especially in the high concentration ranging from 50 µM to 100 µM. It caused that the proportion of LDH released from Caco-2 cell lines can't reach 100%, set by the amount of LDH released by Caco-2 cells after being exposure to 250 µM SDS, after both 24 hours and 48 hours exposure to highest concentration of WIG and 6-PN.

As shown in **Figure 6**, the cell viability (%) calculated based on the florescent signal inside Caco-2 cells was various and instable after being exposed to 6-PN, which would be attributed to the overlapping Caco-2 cell line in some wells. Its causes were diverse, and the main cause would be the detachment of cell lines in small scares during pipette and washing process. The 3×3 circle multiple reads with 750 µm border could be used during the measurement to get an average of four times reads in separate areas in each well, which could get rid of the higher collective fluorescence in the limited area with lumps in further CFDA-AM assay towards differentiated Caco-2 cell lines.

Based on the discussion about the low recovery efficiency of WIG extracted from medium and cells by MeOH and ACN with 0.1% (*v/v*) FA, the high lipophilicity of WIG and the loss of cells would be the main problems. Increasing the volume of MeOH and ACN (containing 0.1% (*v/v*) FA) in cell extraction from 200 µL to 300 µL would enhance the diffusion rate between cells and solvents to achieve a higher extraction efficiency from cells. Meanwhile, to prevent the loss of WIG during the pipetting, it's better to pull out the supernatant from the insert on the tissues instead of pipetting it out. In addition, samples of transport medium in the washing step are supposed to be measured in LC-MS/MS to confirm whether WIG was dissolved and washed out in the transport medium.

In LC-MS measuring, the main task is to optimize the method to make sure the signal is stable and robust during the whole measuring. From the previous optimization result (**Table 8** in **Appendix A.2**), there was a crossover problem in the blank between each turn. To deal with this problem, the highest concentration 10 µM in the calibration curve should be removed. Meanwhile, adding two blanks between each batch of samples would reduce the possibility of cross-over. If the issue would not be tackled after that, the following solutions, like adding an internal standard or measuring the calibration curve several times for correction, could be considered.

5. Conclusion

In summary, WIG showed higher basal cytotoxic potency than 6-PN towards proliferating Caco-2 cells, it may attribute to its higher affinity with cell membranes induced by its higher lipophilicity. The unclear relationship between the concentration range of WIG and 6-PN that exert no adverse effect towards proliferating Caco-2 cells and differentiated Caco-2 cells posed the main challenge in this research. The nominal concentration range used in further Caco-2 cell permeability assay should be determined by exposing differentiated Caco-2 cell lines to WIG and 6-PN and measured their highest non-toxic concentration in the Alamar Blue assay, CFDA-AM assay and BCA assay. LDH assay was not suitable for the further experiment to measure the basal cytotoxicity of WIG and 6-PN towards differentiated Caco-2 cells. Due to their low water-solubility, the percentage of LDH released from cells didn't reach 100% after being exposed to 100 µM WIG and 6-PN, which led to their EC_{50} value obtained in LDH assay being too high to compare with the value obtained in the rest of three assays. Meanwhile, the unstable signal encountered in CFDA-AM assay could be solved by applying the multiple reads during measuring the fluorescent signal inside the cells. The lower IC_{50} and IC_{10} values observed in the BCA assay may be attributed to an interaction between prenylated phenolics and cellular proteins, leading to an underestimation of protein concentration in Caco-2 cells. In addition, the recovery efficiency of WIG was lower than 90%, which may cause by the loss of cells when pipetting out the supernatant from the glass insert, and the less efficiency on extracting it from cells because of its high lipophilicity. To tackle with these problems, increasing volume of organic solvents in cell extraction step and pulling out the supernatant in the glass insert instead of pipetting would be necessary. Besides, the cross-over effect in LC-MS/MS measuring may result in the decreasing peak area over time. To deal with this problem, it's important to remove the 10 µM WIG standard in calibration curve and add two blanks between each batch of samples to wash away the WIG left on the needle from the last measurement.

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Appendix A.

A.1. The detailed dilution procedure in each experiment

A.1.1 The dosing stock solution of WIG and the WIG standards used in calibration curve

This part introduces the detailed protocol about making wighteone standard for calibration curve.

- Make 10mM dosing stock solution of WIG in DMSO by taking 15 uL of master stock solution (20 mM) in DMSO into a 1.5mL glass vials with insert and add 15uL DMSO. Pipette up and down to mix. Cap and store at -20°C.
- Make 50 μM dosing stock solution of WIG in MeOH:T M=2:1 (*v/v*) by adding 10 μL of 10mM WIG in 1990 μL MeOH: TM=2:1 (*v/v*) in 4 mL glass vial. Cap, vortex and store at -20°C
- For WIG standards used in making calibration curve, the concentration series is shown below. The analytical solvent is MeOH: TM=2:1 (*v/v*). All the standards were stored at 4°C.
  - 10 μM: 200 μL of 50 μM solution in 800 μL analytical solvent in 1.5 mL LC-glass vial
  - 3.33 μM: 300 μL of 10 μM solution in 600 μL analytical solvent in 1.5 mL LC-glass vial
  - 1.11 μM: 300 μL of 3.33 μM solution in 600 μL analytical solvent in 1.5 mL LC-glass vial
  - 0.370 μM: 300 μL of 1.11 μM solution in 600 μL analytical solvent in 1.5 mL LC-glass vial
  - 0.123 μM: 300 μL of 0.370 μM solution in 600 μL analytical solvent in 1.5 mL LC-glass vial
  - 0.0412 μM: 300 μL of 0.123 μM solution in 600 μL analytical solvent in 1.5 mL LC-glass vial

A.1.2 The dilution of WIG and 6-PN in cytotoxicity assay

The Caco-2 cells was exposed to WIG and 6-PN with concentration ranging from 0.78 μM to 10 μM. They were diluted by the 20 mM master stock solution of WIG and 6-PN. The dilution procedure is shown in **Figure 10** below.

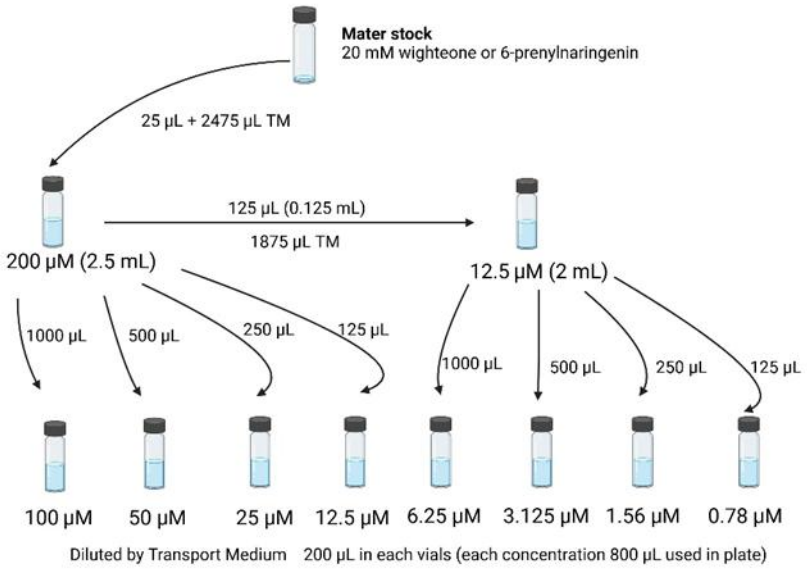

Figure 10. The dilution procedure for the wighteone and 6-prenylnaringenin with concentration ranging from 0.78 μM to 10 μM used in the cytotoxicity experiment.

A.1.3 Positive control (SDS) used in cytotoxicity experiment

Make 50 mM master stock solution of Sodium dodecyl sulfate (SDS) by weighing 14.42 mg SDS to 1.5mL LC vial and adding 1 mL MeOH. The diluted factor between dosing stocks was 2 times, and the one between dosing stocks and its corresponding dosing solution was 200 times. The dilution of dosing stocks and dosing solutions is shown in **Figure 11**.

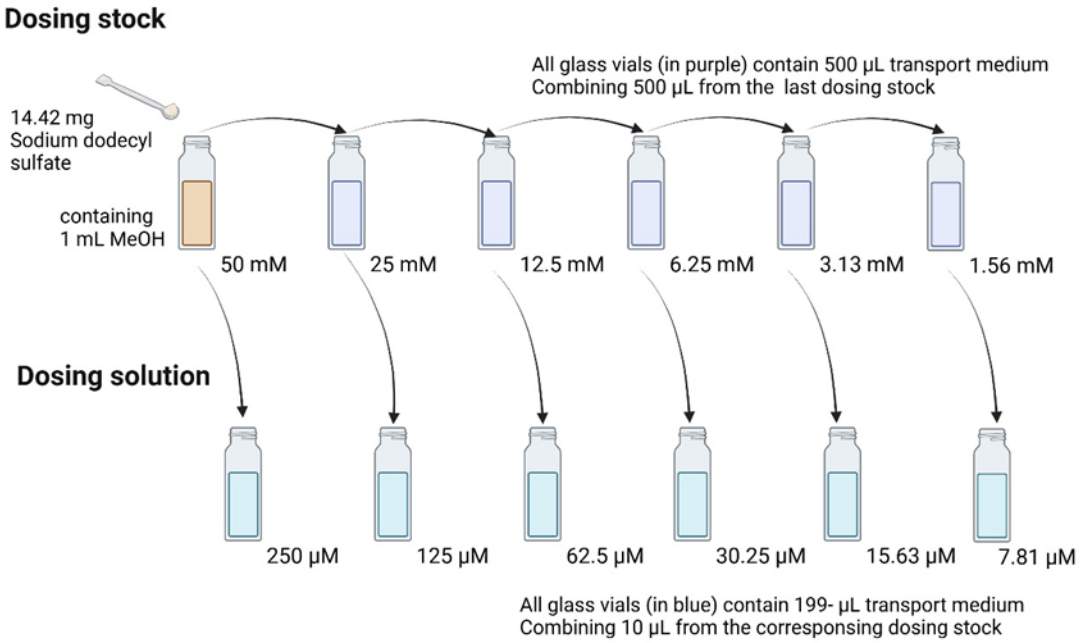

Figure 11. The dilution procedure for positive control Sodium dodecyl sulfate (SDS). The glass vials in orange and purple are dosing stocks. The glass vials in blue are dosing solution used in the 96-well plate.

A.2. The information related to LC-MS/MS measuring

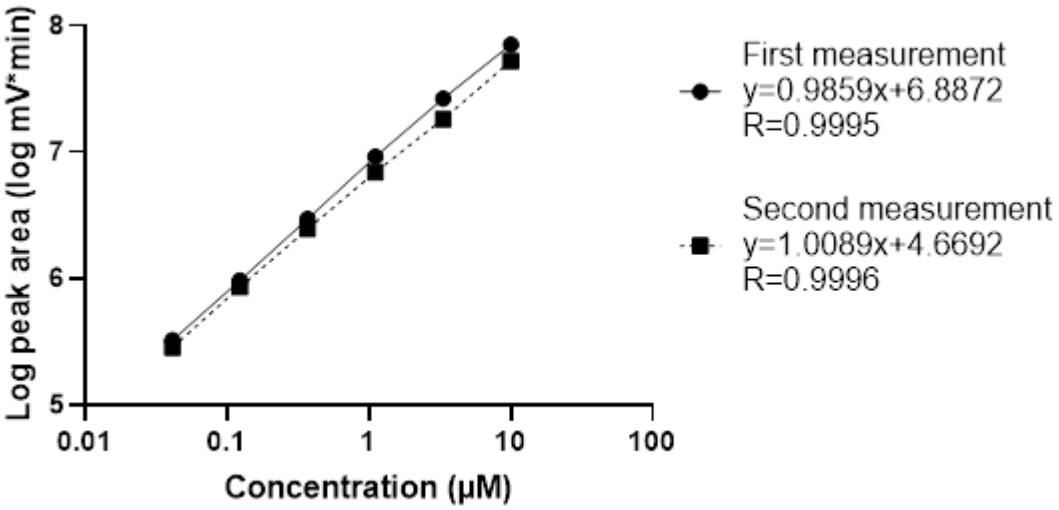

Figure 12. The calibration curves for the measurement before the samples (first measurement) and after samples (second measurement). Their formula and R value was shown in the legend.

Table 8. The peak area (mV*min) of wighteone (WIG) standards with concentration ranging from 0 µM to 10 µM in six turns of measuring in LC-MS/MS.

Concentration (µM)	Peak Area (mV*min)					
	1st	2nd	3rd	4th	5th	6th
0 (Blank)	9,546	60,307	60,843	51,138	56,405	52,973
0.0412	276,131	269,465	260,686	254,372	241,050	247,866
0.123	813,781	761,914	727,358	699,218	677,988	675,146
0.78	2,379,689	2,225,637	2,115,010	2,023,730	1,968,430	1,974,197
1.11	6,646,143	6,278,261	5,849,868	5,779,203	5,616,043	5,707,424
3.33	17,538,513	16,389,281	15,758,722	15,388,444	15,325,826	15,132,593
10	43,446,854	41,647,592	39,820,492	39,494,103	39,046,286	37,894,514

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