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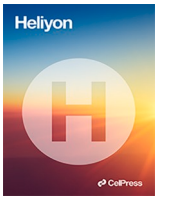

Researchwarticle

Unveiling the potential of medicinal herbs as the source for *in vitro* screening toward the inhibition of Nrf2

Minh Hien Nguyen [a,b,*], Nhi Yen Thi Nguyen [a,b,c], Yi-Siao Chen [d,e], Han Thien Nguyen Le [a], Hoa Thanh Vo [a,b], Chia-Hung Yen [d,e]

[a] *University of Health Sciences, Vietnam National University Ho Chi Minh City, YA1 Administrative Building, Hai Thuong Lan Ong Street, Dong Hoa Ward, Di An City, Binh Duong Province, 75308, Viet Nam*
[b] *Vietnam National University Ho Chi Minh City, Quarter 6, Linh Trung Ward, Thu Duc District, Ho Chi Minh City, 70000, Viet Nam*
[c] *Faculty of Applied Science, University of Technology, Vietnam National University Ho Chi Minh City, 268 Ly Thuong Kiet Street Ward 14, District 10, Ho Chi Minh City, 70000, Viet Nam*
[d] *Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, No.100, Shih-Chuan 1st Road, Sanmin District, Kaohsiung City, 80708, Taiwan*
[e] *National Natural Product Libraries and High-Throughput Screening Core Facility, Kaohsiung Medical University, No.100, Shih-Chuan 1st Road, Sanmin District, Kaohsiung City, 80708, Taiwan*

A R T I C L E I N F O

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A B S T R A C T

Background: Drug resistance is one of the leading causes attributed to the failure of cancer treatment by chemotherapy. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor regulating gene expression in cell defense against oxidative stress or hazardous factors. Taking advantage of this feature, Nrf2 also serves as the bodyguard for both normal and cancer cells. Many pieces of evidence have reported that inhibiting Nrf2 activity in cancer cells can reverse chemotherapy drug resistance. In addition, secondary metabolites from medicinal plants have been reported to inhibit Nrf2 activity in the *in vitro* study. This study aimed to preliminarily investigate fractions from medicinal herbs that inhibit Nrf2 activity in Huh7 liver cancer cells, thereby establishing a basis for subsequent isolation and extraction processes.

Materials and methods: Sub-fractions from five medicinal plants have been evaluated the Nrf2 inhibitor activity on Huh7 cells through luciferase-reported genes assay. Thin-layer chromatography (TLC) was also performed to quantify the extracts' main phytochemistry components. Combining the half-maximal inhibitory concentration (IC_{50}) and half-maximal cytotoxicity concentration (CC_{50}) enables us to determine which extracts have the potential for further isolation steps.

Results: Ten over 30 crude extracts and sub-fractions showed the inhibition of Nrf2 activity with the percentage ranging from 30 to 97 %. The methanol and *n*-hexane sub-fractions from *Helicteres hirsuta* Lour. leaves showed the strongest inhibition ability on Nrf2 activity with the $IC_{50} = 20.98 \pm 3.67$ and 42.22 ± 2.10 μg/mL, respectively. The TLC results showed the presence of steroids and terpenoids in the promising sub-fractions.

Conclusions: Combining the TLC results with the *in vitro* screening on Nrf2 activity screening of medicinal plants, the outcomes suggest the steroids and terpenoids in the methanol extract and

* Corresponding author. University of Health Sciences, Vietnam National University Ho Chi Minh City, YA1 Administrative Building, Hai Thuong Lan Ong Street, Dong Hoa Ward, Di An City, Binh Duong Province, 75308, Viet Nam.

E-mail addresses: nmhien@uhsvnu.edu.vn (M.H. Nguyen), ntynhi.sdh212@hcmut.edu.vn (N.Y.T. Nguyen), wl01420323@gmail.com (Y.-S. Chen), lnthan.d2019@medvnu.edu.vn (H.T. Nguyen Le), chyen@kmu.edu.tw (C.-H. Yen).

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hexane sub-fraction from *Helicteres hirsuta* Lour. leaves show promise towards inhibiting Nrf2 activity in liver cancer cell lines without toxicity in the normal cells.

1. Introduction

Cancer resistance has become a significant concern worldwide, as nearly 90 % of cancer-related deaths are directly or indirectly related to chemotherapy resistance [1]. Resistance to chemotherapy refers to a phenomenon in which cancer cells develop and resist the effects of chemotherapy agents, resulting in reduced effectiveness or complete loss of anticipated treatment efficacy [2]. Drug resistance can be categorized based on the timing of its development, either as intrinsic or acquired resistance [3]. Drug resistance in cancer cells can emerge through various mechanisms, such as genetic factors [2], enhanced drug efflux [4,5], growth factors [6], and increased DNA repair ability [7]. In response to this challenge, one of the recent global research endeavors is exploring resistance mechanisms and discovering natural compounds that could be involved in drug-resistance phenomena.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor contributing to cellular defense mechanisms against oxidative stress and inflammation, simultaneously supports maintaining homeostasis, and inhibits cell aging [8,9]. Nrf2 acts as a cellular protector in both normal cells and cancer cells because Nrf2 can regulate the expression of numerous genes associated with cell protection against carcinogenesis upon exposure to free radicals and oxidative stress [10]. However, in cancer cells, Nrf2 plays a critical regulatory role in cells and is intricately involved in cancer initiation, progression, and metastasis [11]. Indeed, overexpression of Nrf2 in cancer cells has been shown to promote antioxidant genes, namely NAD(P)H quinone oxidoreductase 1, superoxide dismutase or peroxidases enhancing their resistance to chemotherapeutic agents including platinum-based chemotherapeutics [12–15].

Direct or indirect inhibition of Nrf2 expression was reported to enhance the cancer cell's sensitivity to chemotherapy drugs and reverse drug resistance [16]. Therefore, inhibition of Nrf2 expression is currently considered a promising target in strategies to combat cancer drug resistance.

Medicinal herbs have long been used in medicine for years due to their various pharmaceutical effects from secondary metabolites. Several studies have proven that some medicinal extracts can reverse the resistance in cancer cells or resensitize cancer cells to drugs by inhibiting Nrf2 expression. Luteolin, a flavone rich in carrots, peppers, cabbages, and apple skins, was demonstrated to inhibit Nrf2 expression levels significantly. Co-administered luteolin with actinomycin D reduced 34 % Nrf2 mRNA levels after 30 min and 43 % after 1.5 h in human lung carcinoma A549 cells [17]. Luteolin was also reported to enhance chemosensitivity and regulate the stemness in breast cancer by the Nrf2-mediated pathway [18]. Procyanidins isolated from the *Cinnamomi cortex* were demonstrated to possess anti-proliferative properties in lung and prostate cancer cells and inhibit both Nrf2 expression and the activity of Nrf2-regulated enzymes [19,20]. Brusatol, a compound extracted from *Brucea javanica*, has shown the ability to overcome drug resistance by inhibiting Nrf2 activation in various types of cancer [21]. Research published by Ren et al. showed that brusatol inhibits the Nrf2-mediated defense mechanism in lung cancer cells [22]. Many studies also reported the ability of brusatol to restore Nrf2 activity in cells, induce other signaling pathways, and cause growth-inhibitory or cell-apoptotic effects [23,24]. Although natural compounds have revealed potential to bolster cancer drug resistance by suppressing Nrf2 expression, the Nrf2 inhibitors and inhibiting mechanism remains a limitation for research and clinical applications [25], as some of these compounds may exhibit toxicity toward healthy cells alongside their Nrf2 inhibition ability [25–28]. Thus, further research on Nrf2 inhibitors from medicinal plants and their cytotoxicity to normal cells should be carefully considered and evaluated.

In the present study, the authors conducted experiments with methanol extracts from medicinal plants that exhibited potent inhibition of Nrf2 activity, as reported in a previous publication [29]. To yield the sub-fractions, the methanol extracts were dispersed in distilled water then fractionated with *n*-hexane, chloroform, ethyl acetate, *n*-butanol. The sub-fractions were screened for cytotoxicity on Huh7 and HaCaT cells and the relative Nrf2 activity on Huh7. HaCaT cells were tested simultaneously to investigate the toxicity of sub-fraction on normal cells, especially skin cells, due to their common reaction to the toxicity observed on the skin in chemotherapy treatment [30]. After that, potent sub-fractions were determined the half-maximal inhibitory concentrations (IC_{50}) in the relative Nrf2 activity and the half-maximal cytotoxicity concentrations (CC_{50}) in Huh7 cells. The authors also performed thin-layer chromatography to qualify the main compounds in the sub-fractions.

2. Materials and Methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM, product No. A1443001), Gibco™ Fetal Bovine Serum (product No. 10437-028), penicillin-streptomycin (product No. 15140-122), non-essential amino acids (product No. 11140-035), L-Glutamine (product No. 25030-081), and alamarblue (product No. DAL1100) were purchased from Thermo Fisher Scientific, USA. Methanol (CAS: 67-56-1), *n*-hexane (CAS 110-54-3), chloroform (CAS: 67-66-3), ethyl acetate (CAS 141-78-6), *n*-butanol (CAS: 71-36-3), toluene (CAS: 108-88-3), ammonia solution (CAS: 1336-21-6), and formic acid (CAS: 64-18-6) were purchased from Xilong Scientific Co., Ltd., China. Luciferase assay system (product No. E1501) and cell culture lysis 5x (product No. E1531) were purchased from Promega, USA. Dimethyl sulfoxide (DMSO, product No. 13407-45) was acquired from Nacalai Tesque Inc., Japan. 2-aminoethyl diphenylborinate (NP, CAS: 524-95-8) was purchased from Macklin, China. Polyethylene Glycol PEG 400 (CAS: 25322-68-3) was purchased from Henan Jinhe Industry Co., Ltd., China.

2.2. Instrumentals

Biological safety cabinet (Sanxiong Technology, Taiwan), RE300 Vacuum Evaporator (Stuart, UK), Analytical balance (Ohaus, USA), Synergy HT Multi-Mode Reader (Bio-Tek Instruments Inc, USA); NuAire CO_2 incubator (NuAire, USA), WFH-203B thin-layer chromatography lamp (China).

2.3. Medicinal plant collection, preparation, and extraction

Based on our previous screening results for the potent Nrf2 inhibitor extracts from Vietnamese medicinal plants [29], we collected five medicinal plants exhibiting significant capability in inhibiting Nrf2 activity, including *Piper sarmentosum* Roxb. roots (RPS), *Phyllanthus amarus* Schumach. & Thonn. stems (SPA), *Zingiber zerumbet* (L.) Roscoe ex Sm. rhizomes (RZZ), *Helicteres hirsuta* Lour. leaves (LHH), and *Oroxylum indicum* (L.) Kurz stems (SOI). The medicinal collection and methanol extract procedure was carried out as previously published [29]. Briefly, 25.0 g of each medicinal powder was soaked with 400 mL of methanol for three days. The resulting solution was filtered using Newstar 102 filter paper and evaporated under vacuum conditions to obtain the methanol extract. The methanol extract was then subjected to sub-fractions, as described in Fig. 1. The MeOH extracts were dispersed in distilled water and then fractionated with *n*-hexane (Hex), chloroform ($CHCl_3$), ethyl acetate (EA), and *n*-butanol (BuOH). The sub-fraction after extraction with BuOH is called the remaining water extract (rDW). Solvent removal was done using a vacuum evaporator to obtain the respective extracts. These extracts were then stored at 4 °C to perform further experiments.

2.4. Cell culture

Huh7 HCC cell line with the reporter luciferase gene and HaCaT cell line were kindly provided by Professor Chia-Hung Yen (Kaohsiung Medical University, Taiwan) [31]. The Nrf2 activity on Huh7 cancer cells was established based on the transformation of a vector containing a luciferase reporter gene Luc2p. The relative activity of Nrf2 was determined by lysing the transfected Huh7 cells, then determining the luminescent signal generated when reacting with the luciferin substrate. The Huh7 cells were cultured in DMEM, including 10 % of fetal bovine serum (Gibco), penicillin (100 U/mL), streptomycin (100 μg/mL), nonessential amino acids (0.1 mM), and L-glutamine (2 mM). The HaCaT cells were cultured in DMEM with 10 % heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). The cells were maintained in a humidified incubator at 37 °C and an atmosphere of 5 % CO_2.

2.5. Nrf2 activity screening assay

2.5.1. Cell viability assay

The cytotoxicity was determined following the manufacturer's protocol [32]. The Huh7 and HaCaT cells (10^4 cell/well) were seeded in 96-well plates and subsequently exposed to the extract in DMSO (100 μg/mL) for 18 h. After incubation, the old media and extract were removed, then 100 μL of fresh media with 10 % of alamarblue reagent was added and continuously incubated for 4 h. The fluorescence of resazurin in reduced alamarblue was measured using the Synergy HT Multi-Mode Reader (BioTek, Winooski, VT, USA) with excitation and emission light wavelengths of 560/590 nm. DMSO (1 %) was used as the negative control. The resulting formula

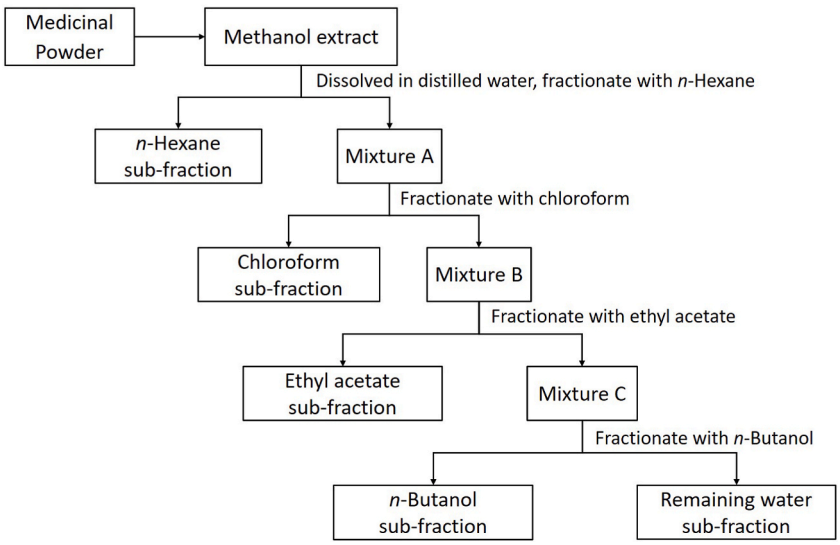

Fig. 1. Procedure of fractionating the crude methanol extracts from five medicinal plant powder. X: Medicinal plants including LHH, RPS, RZZ, SOI and SPA; MeOH: methanol; Hex: n-hexane; $CHCl_3$: chloroform; EA: ethyl acetate; BuOH: n-butanol; rDW: remaining water extract.

used for determining cell viability is as follows:

$$\%\ \text{cell viability} = \frac{F_{sample}}{F_{DMSO}} \times 100\% \quad (1)$$

Where: F is the resazurin fluorescence in alamarblue.

2.5.2. Luciferase reporter gene assay

The relative Nrf2 activity was determined based on luciferase reporter gene assay as described by Wu et al. with minor revision [33]. After determining cell viability as described in Section 2.5.1., the cells were harvested for luciferase activity measurements following the manufacturer's protocol (Promega Corporation, Madison, WI, USA). The relative Nrf2 activity was calculated by normalizing the luciferase activity with cell viability. The negative control well containing 1 % DMSO was referred to a relative Nrf2 activity of 100 %. Luteolin (50 μM) was used as a positive control to evaluate relative Nrf2 activity on the Huh7 cell line. Nrf2 activity of experimental wells was determined as follows:

$$\%\ \text{relative Nrf2 activity} = \frac{F_{sample}/V_{sample}}{F_{DMSO}/V_{DMSO}} \times 100\% \quad (2)$$

Where: F: fluorescence of the reaction of luciferase protein with luciferin.

V: number of survival cells, determined through the fluorescence of resazurin in alamarblue.

All experiments were performed three times, and results are expressed as mean ± standard deviation.

2.6. Determination of 50 % inhibition concentration and 50 % cytotoxicity concentration

Based on the screening results, the potent sub-fractions were conducted to determine the half-maximal inhibitory concentration of Nrf2 activity (IC_{50}) and 50 % cytotoxicity concentration (CC_{50}) on Huh7 cells. To determine CC_{50}, the Huh7 cells were seeded into 96-well plates (3000 cells/well) overnight. The cells were treated with a series of concentrations of extract diluted in DMSO for 72 h at 37 °C and in the atmosphere with 5 % CO_2. Then, the culture medium and medicinal extract were removed, and each well was replenished with 100 μL of fresh culture medium and 10 μL of alamarblue solution (1 mg/mL). The plates were then incubated for an additional 4 h. Fluorescence emitted by the reduced alamarblue was measured in the supernatant using a Synergy HT Multi-Mode Reader (BioTek, Winooski, VT, USA) with excitation/emission wavelengths set at 560/590 nm. Cell viability percentage was calculated using the following formula (1). The CC_{50} value was determined based on the non-linear regression curve between the log (concentration) and the percentage of viable cells [34].

To determine IC_{50}, a similar procedure was carried out to screen the ability to inhibit Nrf2 activity on the Huh7 cell line. In brief, after being seeded in 96-well plates, the Huh7 cells were treated with a series of concentrations of sub-fractions in DMSO (ranging from 6.25 to 500.0 μg/mL) to determine the relationship between dose and activity of Nrf2. The IC_{50} value was determined based on the non-linear regression curve between the log(concentration) and relative activity of Nrf2 [34].

2.7. Thin-layer chromatography

Thin-layer chromatography was performed to identify the main phytochemistry components in the sub-fractions. Specifications for implementing TLC were specified in Vietnam Pharmacopoeia V (2018) [35]. The extract was dispensed in MeOH or $CHCl_3$, depending on the phytochemistry component examined. Approximately 5 μL of the diluted extract was spotted onto a TLC F254 plate (Merck, German). The plate was naturally dried at room temperature and then developed in a 10 × 10 cm chamber with a suitable mobile solvent. All the mobile solvents and reagents used are described in Table 1. After development, the plate was air-dried for 10 min, and the spots were observed at three conditions, including visible light, 254 nm, and 365 nm before and after spraying with the reagents. The parameters, including shape, color, number of spots, and the retention factor (R_f) are documented. The R_f was determined based on spots with color reaction with specific reagents corresponding to the phytochemistry and was calculated following the formula:

$$R_f = a/b$$

Where a is the distance the spot runs on the TLC plate (cm);

b is the distance the solvent runs on the TLC plate (cm).

Table 1
Solvent systems and reagents.

Phytochemical component	Mobile solvent	Ratio	Reagent	Color reaction
Alkaloids	Toluene: EA: NH_4OH	75 : 24: 1	Dragendorff	Yellow → Orange
Anthranoids	EA: MeOH: H_2O	81 : 11: 8	NH_4OH	Pink → Reddish brown
Flavonoids	EA: H_2O: HCOOH	92 : 5: 3	NP/PEG	Yellow or green (UV 365 nm)
Terpenoids	$CHCl_3$: MeOH: H_2O	65 : 35: 10	Vanillin Sulfuric	Pink → Purple
Phenolics	$CHCl_3$: EA: MeOH	50 : 30: 20	$FeCl_3$ 5 %	Green → Blackish green → Black

2.8. Statistical analysis

The raw cell viability data and relative Nrf2 activity were examined and exported using Gen5 software (Version 2.04, BioTek®, USA). Data analysis was performed using Microsoft Office Excel 2016. All graphs were illustrated with Graph Pad Prism (version 9.5.0.730, Dotmatics, USA). All experiments were performed in triplicate, and results were presented as mean ± standard deviation.

3. Results

3.1. In vitro screening on regulative Nrf2 activity and cytotoxicity of sub-fractions

The determination of the ability to inhibit the relative activity of Nrf2 on Huh7 cells and the toxicity of the extract on Huh7 and HaCaT cells results are shown in Table 2. In this study, the HaCaT cells were experimented with simultaneously with the Huh7 cells to investigate the effect of sub-fraction extracts on both normal and cancer cells. Ten over 30 extracts exhibited moderate to solid inhibition of relative Nrf2 activity on Huh7 cells. Notably, RZZ-MeOH extract demonstrated remarkable efficacy in inhibiting over 97.6 % of Nrf2 activity within Huh7 cells, coupled with its substantial toxicity, affecting more than 72 % of the Huh7 cells. However, RZZ-MeOH extract showed a cytotoxic ability to normal cells, which kills more than 43 % of HaCaT cells. Sub-fraction Hex from RZZ had a similar ability to inhibit relative Nrf2 activity in Huh7 cells compared with MeOH. RZZ-Hex extracts also demonstrated moderate cytotoxicity to Huh7. However, toxicity observed in HaCaT cells is significantly lower, underscoring the cytotoxicity of RZZ-Hex sub-fraction extract on Huh7 cancer cells. RZZ-$CHCl_3$ and SOI-$CHCl_3$ exhibited similar results, with the ability to inhibit more than 80 % of relative Nrf2 activity and cytotoxicity towards 45 % of Huh7 cancer cells while maintaining HaCaT cell viability at over 80 %. The MeOH, Hex, and $CHCl_3$ sub-fractions from LHH, Hex and EA sub-fractions from RPS, and Hex sub-fraction from SPA showed a

Table 2
In vitro screening of fraction and sub-fractions from five medicinal plants for effectiveness on relative Nrf2 activity on Huh7 and cytotoxicity on Huh7 and HaCaT cells ($N = 3$).

Sample	Sub-fraction	Cell viability (%)		Relative Nrf2 activity (%)
		Huh7[a]	HaCaT[b]	Huh7[a]
LHH	MeOH	68.62 ± 7.22	87.71 ± 5.52	**49.27 ± 17.33**
	Hex	62.81 ± 1.08	95.74 ± 0.25	**38.55 ± 8.09**
	$CHCl_3$	62.33 ± 2.97	94.39 ± 1.19	**57.61 ± 13.86**
	EA	82.60 ± 1.51	97.60 ± 1.72	126.19 ± 19.19
	BuOH	74.00 ± 1.03	98.79 ± 1.34	106.04 ± 7.19
	rDW	84.81 ± 1.32	96.64 ± 4.54	104.8 ± 2.95
RPS	MeOH	81.15 ± 2.87	94.08 ± 6.37	125.53 ± 11.41
	Hex	54.48 ± 3.91	93.51 ± 2.97	**55.70 ± 9.30**
	$CHCl_3$	108.22 ± 4.50	88.41 ± 2.20	72.23 ± 9.92
	EA	115.05 ± 10.70	95.67 ± 1.33	**49.65 ± 4.33**
	BuOH	93.12 ± 3.97	105.26 ± 3.27	95.04 ± 4.11
	rDW	105.70 ± 5.74	108.58 ± 6.96	143.36 ± 10.55
RZZ	MeOH	27.47 ± 5.73	57.45 ± 1.03	**2.41 ± 0.73**
	Hex	42.25 ± 0.35	81.33 ± 5.06	**2.67 ± 0.53**
	$CHCl_3$	55.19 ± 3.56	86.85 ± 4.38	**14.20 ± 5.48**
	EA	82.04 ± 4.91	100.19 ± 5.78	149.20 ± 4.87
	BuOH	92.51 ± 7.91	106.75 ± 6.61	116.01 ± 3.30
	rDW	88.93 ± 3.64	104.18 ± 8.40	92.48 ± 8.06
SOI	MeOH	79.53 ± 6.98	95.97 ± 4.52	117.78 ± 16.85
	Hex	73.40 ± 2.36	89.02 ± 4.65	80.71 ± 9.73
	$CHCl_3$	**49.87 ± 9.60**	80.69 ± 3.07	**17.78 ± 9.80**
	EA	78.42 ± 0.86	95.12 ± 7.21	123.76 ± 7.90
	BuOH	82.48 ± 0.73	96.09 ± 0.91	110.28 ± 2.91
	rDW	95.91 ± 2.77	104.03 ± 0.49	128.54 ± 14.96
SPA	MeOH	78.34 ± 2.05	94.85 ± 0.83	154.71 ± 7.17
	Hex	91.94 ± 0.80	102.83 ± 1.75	**51.56 ± 4.41**
	$CHCl_3$	90.15 ± 1.96	88.76 ± 3.30	109.73 ± 3.87
	EA	80.89 ± 0.33	89.14 ± 2.49	**69.73 ± 2.31**
	BuOH	79.18 ± 1.67	93.57 ± 1.02	84.56 ± 2.85
	rDW	85.47 ± 1.42	96.22 ± 0.55	91.69 ± 3.53
DMSO		100.00	100.00	100.00
LuT (50 μM)[c]		73.36 ± 0.75	–	10.61 ± 0.66

BuOH: n-butanol; $CHCl_3$: Chloroform; EA: Ethyl acetate; Hex: n-hexane; LHH: *Helicteres hirsuta* Lour. leaves; MeOH: Methanol; rDW: Remaining water extract; RPS: *Piper sarmentosum* Roxb. roots; RZZ: *Zingiber zerumbet* (L.) Roscoe ex Sm. Rhizomes; SOI: *Oroxylum indicum* (L.) Kurz stems; SPA: *Phyllanthus amarus* Schumach. & Thonn. Stems.

[a] Huh7, a liver cancer cell line.

[b] HaCaT, a human keratinocyte line.

[c] Luteolin was used as a positive control for Nrf2 inhibition in Huh7.

moderate ability to inhibit the relative Nrf2 activity in Huh7 cells, ranging from 43.59 to 61.45 %. Apart from the RZZ-MeOH extract, all other sub-fractions demonstrated no toxicity towards HaCaT cells, yielding a cell survival rate exceeding 80 %, indicating the specific efficacy of these extracts against Huh7 cancer cells. This outcome suggested the potential of sub-fractions in combating cancer drug resistance by suppressing Nrf2 activity while maintaining non-toxicity toward healthy cells.

3.2. Evaluation of IC_{50} and CC_{50} from the potent sub-fraction extracts

Based on the results from screening relative Nrf2 activity in both total MeOH extract and sub-fractions, ten promising extracts were further tested to determine the concentration that inhibits 50 % of Nrf2 activity and the concentration toxic to 50 % of Huh7 cells. The lower the IC_{50} value, the stronger the extract's ability to inhibit the activity of Nrf2 activity; the lower the CC_{50}, the stronger the extract's toxicity to Huh7 cells. The results are presented in Fig. 2. The LHH-MeOH, LHH-Hex, and RPS-EA exhibited IC_{50} values of 20.98 $\pm$ 3.67, 42.22 $\pm$ 2.10, and 58.29 $\pm$ 3.79 μg/mL, respectively. In addition, their CC_{50} values significantly surpassed their corresponding IC_{50} values, indicating the potential of these extracts in the exploration of compounds capable of inhibiting Nrf2 activity while avoiding cytotoxicity in the cells. Hex extract from RZZ also demonstrated outstanding inhibitory ability against Huh7 cells, with an IC_{50} value of 49.16 $\pm$ 0.56 μg/mL. However, RZZ-Hex extract exhibited superior cytotoxicity towards Huh7 cells, as demonstrated by its CC_{50} value of 15.37 $\pm$ 0.56 μg/mL, three times lower than its IC_{50} value. These findings highlighted the potential of the Hex sub-fraction extract from RZZ in investigating natural compounds for cancer treatment while supporting reduced drug resistance by decreasing Nrf2 activity. RZZ-$CHCl_3$, SOI-$CHCl_3$, and SPA-EA had similar results, with the ability to induce Huh7 cancer cell toxicity being more prominent than the ability to inhibit the activity of Nrf2. This result suggested the possibility of developing targeted cancer drugs from fractionated extracts or compounds isolated from them, with the ability to exert direct toxic effects on Huh7 cancer cells.

3.3. TLC results

The qualitative outcomes of the crude MeOH extract and sub-fractions derived from LHH, RPS, RZZ, SOI, and SPA are described in Table 3. Overall, terpenoid and steroid compounds stood out as the prominent phytochemicals in most of the sub-fraction extracts of all medicinal plants in the study. In particular, the methanol crude and nonpolar sub-fractions of LHH contain these stains within the extracts. The TLC results showed that RPS contained alkaloids in Hex sub-fraction, phenolic compounds in EA, and plenty of terpenoid compounds present in extraction, though there are still no reports about steroid in *P. sarmentosum* except ariel parts [36]. Remarkably, in our study, RZZ contains a variety of secondary metabolites, including flavonoids, alkaloids, phenolics, and terpenoids. The ongoing research on *Z. zerumbet* mainly focuses on aromatic compounds, well-known as zerumbone. Our research indicates that the diversity of compounds in RZZ is a whole new potential research, especially in phenolics and flavonoids isolated from *Z. zerumbet,* including chlorogenic acid, kaemferol, and its glucosides [37,38]. The extracts from SOI showed the presence of alkaloids, anthranoids, flavonoids, phenolics and terpenoids, similar to previously published studies on the chemical composition of SOI [39]. Numerous phenolics were found in the $CHCl_3$ sub-fraction from *O. indicum* stems compared to others extract, suggesting that the inhibitory effect of *O. indicum* stems on Nrf2 activity might be attributed to compounds within the phenolic group. The presence of terpenoids, anthranoids, flavonoids and phenolics were also found in fractions with moderate Nrf2 activity inhibition from SPA, including Hex and EA.

4. Discussion

Our study successfully screened extracts and sub-fractions from five specific medicinal plants for their ability to inhibit Nrf2 activity in Huh7 liver cancer cells. The correlation between IC_{50} and CC_{50} enables us to evaluate the potential of the sub-fractions towards Nrf2

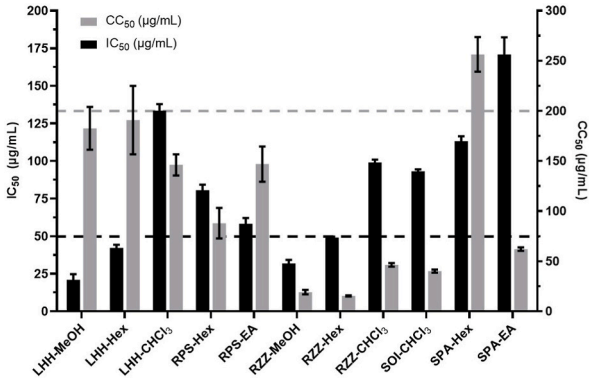

Fig. 2. *In vitro* inhibition activity and cytotoxicity of some sub-fraction extracts against Huh7 cell. $CHCl_3$: Chloroform; EA: Ethyl acetate; Hex: n-hexane; LHH: *Helicteres hirsuta* Lour. Leaves; MeOH: Methanol; RPS: *Piper sarmentosum* Roxb. roots; RZZ: *Zingiber zerumbet* (L.) Roscoe ex Sm. rhizomes; SOI: *Oroxylum indicum* (L.) Kurz stems; SPA: *Phyllanthus amarus* Schumach. & Thonn. Stems (N = 3).

Table 3
TLC results on the main phytochemical components in the extracts from five medicinal herbs.

Sub-fraction	R_f				
	Alkaloids	Anthranoids	Flavonoids	Phenolics	Terpenoids
LHH-MeOH		-	(+++) R_f : 0.62; 0.44 0.10	-	(++++++) R_f: 0.12; 0.32; 0.62; 0.78, 0.81, 0.85
LHH-Hex		-	-	-	(++++++) R_f: 0.13; 0.32; 0.62; 0.78, 0.81, 0.85
LHH-$CHCl_3$		-	-	-	(++++++) R_f: 0.13; 0.32; 0.62; 0.78, 0.81, 0.85
LHH-EA		-	(+++++) R_f : 0.67; 0.62; 0.44; 0.35; 0.10	(++++++) R_f: 0.06, 0.18, 0.41, 0.54, 0.61, 0.67	(+) R_f: 0.62
LHH-BuOH		-	(++++) R_f : 0.62; 0.44; 0.35; 0.10	(+++) R_f: 0.17, 0.27, 0.61	-
LHH-rDW		-	(+++) R_f : 0.62; 0.44; 0.35	-	-
RPS-MeOH	(++) R_f: 0.12, 0.17	-	(+++) R_f : 0.55; 0.47; 0.05	-	(+++) R_f: 0.62, 0.74, 0.82
RPS-Hex	(+++) R_f: 0.11, 0.16, 0.44	-	-	(++) R_f: 0.67, 0.80	(+++) R_f: 0.62, 0.72, 0.82
RPS-$CHCl_3$	-	-	(+++) R_f : 0.78; 0.60; 0.55	(+) R_f: 0.59	(+++) R_f: 0.61, 0.72, 0.82
RPS-EA	-	-	(+) R_f : 0.78	-	(+++) R_f: 0.61, 0.71, 0.81
RPS-BuOH	-	-	(++++) R_f : 0.78; 0.47; 0.55; 0.05	-	(+++) R_f: 0.61, 0.82, 0.87
RPS-rDW	-	-	-	-	-
RZZ-MeOH	(+++++) R_f : 0.18, 0.21, 0.27, 0.32, 0.68	(+) R_f : 0.82	(++++++) R_f : 0.80; 0.75; 0.71; 0.65; 0.57; 0.43	(++++) R_f : 0.40, 0.56, 0.61, 0.72	(+++) R_f : 0.68, 0.79, 0.85
RZZ-Hex	(++++) R_f : 0.18, 0.27, 0.32, 0.68	(+) R_f : 0.82	(++++++) R_f : 0.80; 0.75; 0.71; 0.65; 0.57; 0.43	(++++) R_f : 0.40, 0.56, 0.61, 0.66	(+++) R_f : 0.68, 0.79, 0.85
RZZ-$CHCl_3$	(++++) R_f : 0.18, 0.21, 0.27, 0.32	-	(++++++) R_f : 0.80; 0.75; 0.71; 0.65; 0.57; 0.43	(+++) R_f : 0.61, 0.66, 0.72	(+) R_f : 0.68
RZZ-EA	-	-	(++++++) R_f : 0.80; 0.75; 0.71; 0.65; 0.57; 0.43	(+++) R_f : 0.40; 0.51; 0.56	(+) R_f : 0.68
RZZ-BuOH	-	-	-	-	(+++) R_f : 0.41, 0.59, 0.68
RZZ-rDW	-	-	-	-	-
SOI-MeOH	-	-	(+++) R_f : 0.76; 0.45; 0.15	-	(++++) R_f: 0.63, 0.69, 0.74, 0.84
SOI-Hex	(++) R_f: 0.12, 0.18	-	(+) R_f : 0.15	(+) R_f: 0.68	(+++) R_f: 0.63, 0.74, 0.82
SOI-$CHCl_3$	-	(+) R_f: 0.47	(++++) R_f : 0.80; 0.76; 0.45; 0.15	(+++++) R_f: 0.18, 0.41, 0.51, 0.61, 0.7	(++) R_f: 0.63, 0.82
SOI-EA	-	(+) R_f: 0.47	(+++++) R_f : 0.76; 0.68; 0.45; 0.24; 0.15	(+++) R_f: 0.15, 0.2, 0.41	(++) R_f: 0.63, 0.82
SOI-BuOH	-	(+) R_f: 0.47	(+) R_f : 0.15	(+) R_f: 0.11	(+) R_f: 0.63
SOI-rDW	-	-	-	-	(+) R_f: 0.21
SPA-MeOH	-	-	(++++) R_f : 0.69; 0.60; 0.47; 0.30	+	-
SPA-Hex	-	-	-	-	(++) R_f: 0.87, 0.91
SPA-$CHCl_3$		-	(+++++) R_f : 0.69; 0.60; 0.47; 0.42; 0.30	(+) R_f: 0.56	-
SPA-EA	-	(++) R_f: 0.39, 0.55	(+++) R_f : 0.69; 0.60; 0.47	(+++) R_f: 0.20, 0.29, 0.47	-
SPA-BuOH	-	+	(++) R_f : 0.60; 0.47	+	(+) R_f: 0.44
SPA-rDW	-	-	-	-	-

-Non-detected
+Visible spot on TLC plate, cannot determine R_f (+): Visible spot on TLC plate, the number of "+" mean the number of spots viewed on the plate.

BuOH: n-butanol; $CHCl_3$: Chloroform; EA: Ethyl acetate; Hex: n-hexane; LHH: *Helicteres hirsuta* Lour. leaves; MeOH: Methanol; rDW: Remaining water extract; RPS: *Piper sarmentosum* Roxb. roots; RZZ: *Zingiber zerumbet* (L.) Roscoe ex Sm. Rhizomes; SOI: *Oroxylum indicum* (L.) Kurz stems; SPA: *Phyllanthus amarus* Schumach. & Thonn. Stems;

inhibition. In particular, the methanol extract and Hex sub-fraction of *H. hirsuta* leaves exhibited an outstanding ability to inhibit Nrf2 activity with IC_{50} values lower than 50 μg/mL (20.98 ± 3.67 and 42.22 ± 2.10 μg/mL, respectively). Whereas the CC_{50} values of these fractions are higher than IC_{50} values more than four times, up to 180 μg/mL, and cell viability on HaCaT at 100 μg/mL was nearly 90 %. The EA sub-fraction of *P. sarmentosum* roots describes potent results by inhibiting the relative activity of Nrf2 on Huh7 cells with the relative Nrf2 activity of 49.65 ± 4.33 %. Additionally, the IC_{50} of ethyl acetate sub-fraction of *P. sarmentosum* roots has a much lower value than the CC_{50} (58.29 ± 3.79 compared to 146.87 ± 17.63 μg/mL, as shown in Fig. 2), demonstrating the potential to inhibit Nrf2 at a low concentration without influencing by the cytotoxicity.

Regarding remaining sub-fractions, they showed the predominance of cytotoxic activity with CC_{50} less than 1.5 to two times over the inhibition of Nrf2 on Huh7 cells. More specifically, the methanol extract from *Z. zerumbet* rhizomes showed excellent cytotoxic activity with the CC_{50} value of 19.17 ± 2.22 μg/mL half lower than the IC_{50} value of 31.89 ± 2.38 μg/mL. The study of Ali et al. also reported that methanol extract from *Z. zerumbet* has cytotoxicity against Ehrlich ascites carcinoma cells at low concentrations (IC_{50} = 27.49 ± 2.25 μg/mL) [40]. Furthermore, the sub-fraction in chloroform of *O. indicum* stems and that in ethyl acetate of *P. amarus* stems exhibit cytotoxicity on Huh7 cells with low CC_{50} values of 40.08 ± 1.49 and 62.1 ± 1.71, respectively.

TLC results generally show the diversity in the phytochemical compounds in various sub-fractions (Table 3). The common compounds shared between the extracts with *in vitro* Nrf2 inhibition are steroids and/or terpenoids. In particular, the steroids and/or terpenoids predominate in the MeOH and Hex sub-fractions from *H. hirsuta* leaves, and EA sub-fraction from *P. sarmentosum* roots. The R_f values of the components in MeOH and Hex sub-fractions from *H. hirsuta* leaves show the variety in polarity (Table 3). The low R_f values may indicate steroid or triterpenoid compounds while the higher R_f may correspond to volatile terpenoid compounds.

Currently, the studies are mainly focused on flavonoids to seek novel Nrf2 inhibitors, while the effects of terpenoids inhibiting Nrf2 activity in cancer cells have not been appropriately exploited despite the structural diversity of terpenoids [25]. Several studies have revealed that terpenoids can affect cancer cells through many different pathways [41], including regulation of PI3K, MAPK, and inducing apoptosis pathway in cancer cells [42,43]. Brusatol and brucein, two degraded triterpene compounds extracted from the seeds of *Brucea javanica*, have been identified as Nrf2 inhibitors regulating Nrf2 activity [22,44]. Recently, stigmasterol was demonstrated to be a potential candidate for cancer treatment because it sensitizes endometrial cancer cells to cisplatin by significantly inhibiting Nrf2 activity and enhancing the effect of cisplatin on cellular growth, migration, and invasion [45].

In addition to the steroids and terpenoids, alkaloids and phenolics are present in other sub-fractions exhibiting potent inhibition of Nrf2 activity, such as Hex sub-fraction from *P. sarmentosum* roots, Hex and $CHCl_3$ sub-fractions from *Z. zerumbet* rhizomes. Phenolic and alkaloids are potential compounds that can resensitize cancer cells to chemotherapy drugs or reverse cancer drug resistance through the ATP-Binding Cassette transporter pathway in cancer cells [46].

Interestingly, the simultaneous presence of terpenoids, alkaloids, and phenolics in the methanol extract of *H. hirsuta* leaves, together with the lowest IC_{50} among their sub-fractions, suggest the potential synergistic effects of various compounds in the extracts. This highlights the advantage of natural compounds in cancer treatment in their ability to impact multiple targets and mechanisms while causing less toxicity [47]. The Nrf2 inhibitor compounds found recently impact upstream and downstream signaling pathways of Nrf2 molecular expression, including autophagy, PI3K/AKT pathway, and oncogenic interaction [25,48].

5. Conclusion

Nrf2 is a promising target for anti-cancer treatment, especially in chemotherapy resistance in cancer with overexpression of Nrf2. Our findings indicated the potential of steroids and terpenoids from methanol and *n*-hexane sub-fraction of *H. hirsuta* to overcome anticancer-drug resistance by suppressing Nrf2 activity. At present, the studies of Nrf2 inhibitors have been significantly limited. In the future, it is necessary to have insight into the Nrf2 activity regulation mechanism along with structure-activity relationship research on the natural Nrf2 inhibitors.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Minh Hien Nguyen: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Nhi Yen Thi Nguyen:** Visualization, Methodology, Investigation, Formal analysis. **Yi-Siao Chen:** Methodology, Investigation, Formal analysis. **Han Thien Nguyen Le:** Writing – original draft, Investigation, Formal analysis, Data curation. **Hoa Thanh Vo:** Writing – original draft, Formal analysis. **Chia-Hung Yen:**

Writing – original draft, Methodology, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38411.

List of abbreviation

BuOH	*n*-butanol
CC_{50}	Half-maximal cytotoxicity concentration
$CHCl_3$	Chloroform
EA	Ethyl acetate
Hex	*n*-hexane
IC_{50}	Half-maximal inhibitory concentration
LHH	*Helicteres hirsuta* Lour. leaves
MeOH	Methanol
Nrf2	Nuclear factor erythroid 2-related factor 2
TLC	Thin-layer chromatography
SOI	*Oroxylum indicum* (L.) Kurz stems
SPA	*Phyllanthus amarus* Schumach. & Thonn. stems
rDW	Remaining water extract
RPS	*Piper sarmentosum* Roxb. roots
RZZ	*Zingiber zerumbet* (L.) Roscoe ex Sm. rhizomes

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