Original Article

JHSMR Journal of Health Science and Medical Research

Antioxidant, Anti-Inflammatory, and Cytotoxic Activities Against Drug-Sensitive and Drug-Resistant Cancer Cells of the Extracts of *Clausena excavata*, *Millettia pachycarpa*, and *Uvaria grandiflora*

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Received 22 May 2025 ● Revised 24 July 2025 ● Accepted 18 August 2025 ● Published online 4 March 2026

Abstract:

Objective: This study aimed to analyze the chemical profiles of bioactive compounds in extracts from different parts of *Clausena excavata (C. excavata)*, *Millettia pachycarpa (M. pachycarpa)*, and *Uvaria grandiflora (U. grandiflora)*, and to examine their antioxidant, anti-inflammatory, and cytotoxic effects on drug-sensitive and -resistant cancer and normal cells.

Material and Methods: Ethyl acetate extracts from *C. excavata* fruits (FCE), *M. pachycarpa* roots and leaves (RMP and LMP), and *U. grandiflora* twigs and leaves (TUG and LUG) were analyzed for total phenolic and flavonoid content and the chemical profiles of bioactive compounds. Antioxidant activities were determined using various methods. Anti-inflammatory properties were investigated in lipopolysaccharide-stimulated RAW264.7 cells, and cytotoxicity was evaluated in doxorubicin-sensitive and -resistant leukemic cells (K562 and K562/adr), breast cancer cells (MCF-7 and MCF-7/adr), and normal cells (peripheral blood mononuclear cells; PBMCs).

Results: The extracts contained bioactive compounds, including carbazole alkaloids, xanthones, and coumarins in FCE; isoflavonoids, coumarins, and rotenoids in RMP and LMP; and alkaloids, cyclohexenes, and flavonoids in TUG and LUG.

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J Health Sci Med Res 2026;44(4):e20261318
doi: 10.31584/jhsmr.20261318
www.jhsmr.org

TUG, RMP, and LMP exhibited the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, whereas RMP demonstrated the most potent 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and superoxide radical scavenging activities. LUG showed strong metal-chelating activity (MCA), and LMP showed superior ferric ion reducing antioxidant power (FRAP). FCE exhibited notable anti-inflammatory activity, whereas RMP showed significant cytotoxicity against MCF-7 and MCF-7/adr cells, with minimal toxicity to PBMCs. TUG also proved effective against drug-resistant leukemia cells.

Conclusion: These findings highlight the potential of these plants for use in dietary supplements and cancer treatments, especially for drug-resistant cancers.

Keywords: anticancer, anti-inflammation, antioxidant, *Clausena excavata*, *Millettia pachycarpa*, *Uvaria grandiflora*

Introduction

Medicinal plants are widely used for disease prevention, treatment, and other health purposes because of their low toxicity and few adverse effects[1]. The biologically active constituents are polyphenolic compounds and flavonoids, which exhibit various pharmacological activities, such as antioxidant, anti-inflammatory, antimicrobial, and anticancer activities[2,3]. Previous studies have shown that the three abovementioned medicinal plants contain chemical components that are key to their biological activities. For example, the roots and fruits of *C. excavata* contain coumarin and carbazole alkaloids that exhibit 2,2′-diphenyl-1-picrylhydrazyl (DPPH) antioxidant activity and cytotoxicity against lung cancer (A549), colorectal cancer (SW480), breast cancer (MCF-7), and erythromyelogenous leukemia (K562) cells. They also inhibit α-glucosidase enzyme activity and increase glucose uptake into cells[4-7]. Additionally, the seeds of *M. pachycarpa*, which contain isoflavone and chalcone compounds, possess anti-inflammatory effects in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages and show cytotoxicity against hepatocellular carcinoma (HepG2), colon cancer (C26), lung cancer (LL2), and skin cancer (B16) cells[8,9]. The roots and leaves of *M. pachycarpa*, which contain rotenoid and isoflavonoid compounds, exhibit cytotoxicity against lung cancer (A549), colon cancer (SW480), and leukemic (K562) cells. These compounds also inhibit the activity of α-glucosidase and α-amylase enzymes for antidiabetic activity[10]. In addition, the stems and leaves of *U. grandiflora* contain polyoxygenated cyclohexene compounds that demonstrate cytotoxicity against hepatocellular carcinoma (HepG2) and breast cancer (MDA-MB231) cells and inhibit α-glucosidase enzyme activity, resulting in anti-diabetic and anti-obesity activity[11,12]. Moreover, alkaloids isolated from the twigs of *U. grandiflora* show antimicrobial and DPPH-radical-scavenging activities[13].

Cancer is a major public health issue, and it is the leading cause of death among people in Thailand and worldwide. The incidence of cancer in Thailand has been continuously increasing[14]. Cancer treatment with chemotherapy can lead to drug resistance of cancer cells and adverse effects of toxicity to normal cells, resulting in treatment failure[15]. Previous studies have shown that polyphenols and flavonoids can overcome drug resistance in DOX-resistant erythromyelogenous leukemic cells and small-cell lung carcinoma[16-18]. Furthermore, these plant-derived bioactive compounds have been found to be minimally toxic to normal cells[18-20].

However, studies on the chemical profiles of bioactive compounds, antioxidants, anti-inflammation, and the

anticancer activities of the extracts from *C. excavata*, *M. pachycarpa*, and *U. grandiflora* are still limited. In particular, few, if any, studies have investigated the capacity of these three species' extracts to battle drug-resistant cancer cells while also evaluating their safety on normal cells. Therefore, the objective of this study was to analyze the chemical profiles of the bioactive compounds found in extracts from different parts of these plants. The study also aimed to investigate their antioxidant, anti-inflammatory, and cytotoxic activities against both drug-sensitive and drug-resistant cancer cells, while evaluating their impact on normal cells. The results of this study will serve as scientific evidence to confirm the potential of these plants for development into dietary supplements for disease prevention and further into drugs, particularly for the treatment of drug-resistant cancer.

Material and Methods

Chemicals and reagents

RPMI 1640 medium, minimum essential medium, Dulbecco's modified Eagle medium (DMEM), and phosphate-buffered saline (PBS) were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). Penicillin/streptomycin, fetal calf serum (FCS), and Griess reagent were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Lymphoprep™ solution was purchased from Axis-Shield Diagnostics (Dundee, UK). Rezasurin, ethyl acetate, dimethyl sulfoxide, gallic acid, catechin, trolox, ethylenediaminetetraacetic acid (EDTA), potassium ferricyanide, ferric chloride, aluminum chloride, nitro blue tetrazolium (NBT), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), ferric chloride ($FeCl_3$), potassium persulfate, 2,2′-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, ethanol, and methanol (liquid chromatography [LC]-mass spectrometry [MS] grade) were purchased from the Sigma-Aldrich Chemical Company (St Louis, MO, USA) or Merck (Darmstadt, Germany).

Plant materials

The fruits of *C. excavata* were collected from Pakook Village, Bandu Subdistrict, Mueang District, Chiang Rai Province, Thailand (N: 19.9737°, E: 99.8860°) in June 2020. The plants were identified by Professor Surat Laphookhieo. A voucher specimen (MFU-NPR0019) was deposited at the Natural Products Research Laboratory, School of Science, Mae Fah Luang University.

The roots and leaves of *M. pachycarpa* were collected from Pakook Village, Bandu Subdistrict, Mueang District, Chiang Rai Province, Thailand (N: 20.3256°, E: 99.3332°) in March 2018. The plant was identified by the botanist Mr. Martin van de Bult. A voucher specimen (MFU-NPR0207) was deposited at the Natural Products Research Laboratory, School of Science, Mae Fah Luang University.

The twigs and leaves of *U. grandiflora* were collected from Chiang Rai Rajabhat University, Bandu Subdistrict, Mueang District, Chiang Rai Province, Thailand (N: 19.9803°, E: 99.8504°) in July 2019. The plants were identified by Professor Surat Laphookhieo. A voucher specimen (MFU-NPR0169) was deposited at the Natural Products Research Laboratory, School of Science, Mae Fah Luang University.

Preparation of crude extracts

To enrich medium-polarity compounds such as phenolic and flavonoid compounds from these plants, air-dried, crushed fruits of *C. excavata*, roots and leaves of *M. pachycarpa*, and twigs and leaves of *U. grandiflora* were macerated in ethyl acetate. The maceration was completed over a period of 3 days (three times) at room temperature. The liquid extracts were filtered through Whatman No. 1 filter paper and evaporated under vacuum using a rotary evaporator. All the powders of crude extracts were stored in a desiccator until used. The yields of each extract were as follows: 4,500.0 g of dried fruits of *C. excavata* yielded 132.7 g of extract (2.95%), 629.5 and 856.7 g of dried roots

and leaves of *M. pachycarpa* yielded 54.5 and 45.7 g of extract (8.66% and 5.33%), respectively, and 566.3 and 954.8 g of dried twigs and leaves of *U. grandiflora* yielded 16.0 and 49.1 g of extract (2.83% and 5.14%), respectively.

Determination of total phenolic and flavonoid contents

The Folin-Ciocalteu method was used, with slight modifications, to determine the total phenolic content (TPC) of the extracts. All reagents and sample solutions were freshly prepared for the experiments. Folin-Ciocalteu reagent was diluted 1:10 with distilled water; sodium carbonate solution (20% w/v) was prepared in distilled water; and standardized extract solutions and the standard compound, gallic acid, were serially diluted two-fold in 95% (v/v) ethanol. One hundred and twenty microliters of each extract was then mixed with 1,000 µL of Folin-Ciocalteu reagent for 5 min. Then, 1,000 µL of sodium carbonate solution was added to this mixture. The solution was thoroughly mixed and incubated in the dark at 25 °C for 1.5 h. The absorbance was measured at 725 nm using a Sunrise™ Microplate reader (Tecan Group Ltd., Mannendorf, Switzerland). TPC was expressed in mg of gallic acid per mg of extract[21].

The aluminum chloride colorimetric method, with slight modifications, was used to determine the total flavonoid content (TFC) of the extracts. Specifically, 50 µL of each extract was mixed with 300 µL of 5% (w/v) sodium nitrite and 300 µL of 10% (w/v) aluminum chloride, followed by the addition of 4 mL of distilled water. The mixture was incubated at 25 °C for 6 min. Subsequently, 2 mL of 1 M sodium hydroxide solution was added to stop the reaction. The final volume was adjusted to 10 mL using distilled water, and the absorbance at 510 nm was measured after 10 min of incubation under the specified conditions. The catechin concentration in each extract was determined using a regression equation based on the absorbance. The results are expressed as TFC in mg of catechin equivalents per mg of extract[21].

Chemical profile analysis using high-performance liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry

The samples (1 mg of the sample) were dissolved in methanol (LC-MS grade), filtered through a 0.22 µm membrane, and then placed in a vial for analysis. The analysis was conducted using an Agilent 1290 Infinity LC instrument (Agilent, Santa Clara, CA, USA) connected to an Agilent 6540 series quadrupole time-of-flight (QTOF)-MS instrument equipped with an electrospray ionization (ESI) source, a diode-array detector, an automatic sample injector, a degasser, and an Agilent Poroshell 120 EC-C18 column (4.6×150 mm, particle size 2.7 µm). HPLC-ESI-QTOF-MS analysis was performed at 35 °C for the separation of the extracts at a flow rate of 200 µL/min. The mobile phase was water (0.1% formic acid, A) mixed with acetonitrile (0.1% formic acid, B). The elution gradient mode was as follows: 1–10 min, 5% to 17% B; 10–13 min, 17% B, 13–20 min, 17% to 100% B, 20–25 min, 100% B; 25–27 min, 100% to 5% B; 27–33 min, 5% B. The injection volume was 1.0 µL and samples were maintained at 4 °C.

Both negative and positive ion modes were used to confirm the fragment ions in the MS data using energy collision dissociation within the *m/z* ratio range of 50–1,000 at a resolution of 4,000, to establish the chemical profile. The gas temperature was 350 °C, the drying gas flow rate was 12 L min^{-1}, the nebulizer gas pressure was 45 psi, the sheath gas temperature was 250 °C, and the sheath gas flow rate was 12 Arb. Agilent Mass Hunter workstation software B.0800 (Qualitative Analysis, version B.08.00) and the Personal Compound Database and Library (PCDL) were used for LC-MS control and data handling, respectively. Compounds with PCDL scores higher than 80 and lower than 5 ppm were selected for *m/z* verification and MS analyses[22,23].

Determination of DPPH-free-radical-scavenging activity

The DPPH-scavenging assay was performed to assess the ability of the extracts to scavenge DPPH• radicals. A 20 µL sample of various concentrations of the extracts was mixed with 180 µL of 80 µM DPPH solution in ethanol in a pre-filled 96-well plate and incubated for 5 min. The absorbance of the resulting mixture was measured at 492 nm using a Sunrise microplate reader (Tecan Group Ltd.) after 30 min of incubation in the dark at room temperature. Wells containing no extract served as controls, whereas wells containing the extracts served as test wells. The percentage of DPPH radical inhibition by the extracts and the positive control, Trolox, was calculated as follows: inhibition (%) = [($Abs_{492\ nm}$ of control well – $Abs_{492\ nm}$ of test well)/$Abs_{492\ nm}$ of control well] × 100. The concentrations of extracts and Trolox required to inhibit the DPPH radicals by 50% (IC50) were determined by plotting inhibition (%) against extract and Trolox concentrations[21].

Determination of ABTS-free-radical-scavenging activity

$ABTS^{+}$• radicals were generated using a standard method by mixing equal parts of 7 mM ABTS and 2.45 mM potassium persulfate, serving as the substrate and oxidant, respectively. Freshly prepared ABTS solution, incubated for 12 h at room temperature in the dark, was then diluted with ethanol to reach an absorbance of 0.70±0.05 at 734 nm. A 200 µL aliquot of this solution was added to a 96-well microplate containing 20 µL of various concentrations of the extracts. The absorbance of the resulting mixture was measured at 734 nm using a Sunrise microplate reader after 6 min of incubation at room temperature. Wells containing no extract served as controls, whereas wells containing the extract served as test wells. The percentage of ABTS radical inhibition by the extracts and the positive control, Trolox, was calculated as follows: inhibition (%) = [($Abs_{734\ nm}$ of control well – $Abs_{734\ nm}$ of test well)/$Abs_{734\ nm}$ of control well] × 100. The IC50 values of the extracts and Trolox were determined by plotting inhibition (%) against extract and Trolox concentrations[21].

Superoxide-radical-scavenging assay

To evaluate the superoxide-anion-scavenging activity of the extracts, superoxide ions were generated through a photochemical reaction using a combination of riboflavin, methionine, and light irradiation. Activity was determined by measuring the inhibition of purple formazan (NBT^{2+}) formation. The reaction was initiated by preparing a working solution (400 µL) containing riboflavin (30 µg/mL), methionine (30 µg/mL), and EDTA (20 µg/mL), which was then mixed with 100 µL of NBT (400 µg/mL). Various concentrations of the extracts were added to phosphate buffer (0.05 M, pH 7.4). The mixture was exposed to light from a 20 W fluorescent lamp at 25 °C for 25 min, and the amount of formazan produced was measured at 560 nm. The percentage of superoxide radical inhibition by both the extracts and the positive control, catechin, was calculated as follows: inhibition (%) = [($Abs_{560\ nm}$ of control well – $Abs_{560\ nm}$ of test well)/$Abs_{560\ nm}$ of control well] × 100. The concentrations of extract and catechin required to inhibit superoxide radicals by 50% (IC50) were determined by plotting inhibition (%) against extract and catechin concentrations[21].

Determination of metal-chelating activity

Metal-chelating activity (MCA) was assessed by measuring the ability of each extract to bind ferrous ions. Various concentrations of the extracts were prepared and mixed with 25 µL of 2 mM iron (II) chloride and 80 µL of distilled water. Then, 50 µL of 5 mM ferrozine was added to start the reaction, and the mixture was incubated immediately at 25 °C for 10 min. The absorbance of the resulting mixture was measured at 562 nm using a Sunrise microplate reader. Wells containing no extracts served

as controls, whereas wells containing extracts served as test wells. The percentage of metal chelation of both the extracts and the positive control, EDTA, was calculated as follows: MCA (%) = [($Abs_{562\ nm}$ of control well – $Abs_{562\ nm}$ of test well)/$Abs_{562\ nm}$ of control well] × 100. The IC50 values of the extract and EDTA were determined by plotting MCA (%) against extract and EDTA concentrations[21].

Ferric-ion-reducing antioxidant power assay

The antioxidant efficacy of the extracts was determined by testing their ferric ion reducing antioxidant power (FRAP), which measures their ability to convert ferric ions in the ferric-tripyridyl triazine (TPTZ) complex to ferrous-TPTZ. In brief, 1.35 mL of freshly prepared FRAP reagent was mixed with 150 µL of various concentrations of the extracts. These mixtures were then incubated at 37°C for 2 h, and the increase in absorption at 593 nm was measured using a Sunrise™ microplate reader. The absorbance values were plotted against the concentration of ferrous ions in the ethanol solution. The capacity of the extracts to reduce ferric ions is expressed as micromoles of ferrous ion equivalents per milligram of extract (µmol Fe^{2+} equivalents/mg of extract), using a Fe_2SO_4 working solution as a standard curve[21].

Cell models

Cell lines: Murine macrophage leukemic cells (RAW 264.7) were purchased from the American Type Culture Collection (ATCC number: TIB-71TM; Manassas, VA, USA); human doxorubicin-sensitive (K562) and -resistant (K562/adr) erythromyelogenous leukemic cells were a gift from Associate Professor Sawitree Chiampanichayakul, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University (CMU-IBC MTA01); and human doxorubicin-sensitive (MCF-7) and -resistant (MCF-7/adr) breast cancer cells were purchased from iCell Bioscience (Shanghai, China).

Primary normal cells: Human peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat of healthy blood donors who tested negative for infectious markers at Chiangrai Prachanukroh Hospital. This procedure was approved by the hospital's Ethics Committee (CR0033.102/RES/EC66-723).

Cell culture and preparation

Cancer cells were grown in RPMI 1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin and maintained at 37 °C in a humidified atmosphere with 5% CO_2. Subculturing was performed every 3 days. PBMCs were separated from the buffy coat of healthy blood donors using density gradient centrifugation. The buffy coat was diluted with PBS and layered on Lymphoprep™ solution. After centrifugation at 400 × g for 30 min at room temperature, PBMCs were harvested, washed twice with PBS, and centrifuged again at 200 × g for 10 min at room temperature. The cells were immediately used for the cytotoxicity assays.

Determination of anti-inflammatory activity

RAW 264.7 cells were seeded at 4 × 10^4 cells/well in DMEM supplemented with 10% FCS, 2mM L-glutamine, and 100 U/mL penicillin/streptomycin in 96-well plates and incubated with 1 µg/mL LPS at 37 °C in a 95% humidified atmosphere of 5% CO_2 for 1 h. Subsequently, the cells were treated with various concentrations of the extracts and the positive control, indomethacin, for 24 h and nitric oxide (NO) production was measured using Griess reagent. The absorbance of the resulting mixture was measured at 540 nm using an Infinite 200 Pro Microplate Reader (Tecan Group Ltd.). The well containing neither LPS nor the extract served as a control well, the well containing only LPS (no extract) served as the LPS well, and the wells containing LPS and the extracts served as the test wells. The percentage of NO production inhibition was calculated using the following formula: inhibition (%) = [($Abs_{540\ nm}$ of LPS

well – $Abs_{540\ nm}$ of test well)/($Abs_{540\ nm}$ of LPS well – $Abs_{540\ nm}$ of control well)] × 100. The IC50 values were determined by plotting inhibition (%) against extract and indomethacin concentrations[24].

Cytotoxicity assay

The cells (10^4 cells) were seeded into 96-well plates containing 100 µL of RPMI 1640 medium supplemented with 10% FCS and 100 U/mL penicillin/streptomycin. Wells without any extract were used as controls, whereas wells with various concentrations of the extract and the standard chemotherapeutic drug, doxorubicin, served as test wells. All samples were incubated at 37 °C in a 95% humidified atmosphere with 5% CO_2 for 72 h. After incubation, 20 µL of resazurin solution (0.15 mg/mL) was added to each well, and samples were incubated for an additional 4 h. The fluorescence intensity (FI) of the fluorescent product, resulting from resazurin reduction in viable cells, was measured at 590 nm (excitation at 560 nm) using an Infinite 200 Pro Microplate Reader (Tecan Group Ltd.). The percentage of cell viability inhibition was calculated according to the following formula: inhibition (%) = (FI_{590nm} of the test well – FI_{590nm} of the control well) /FI_{590nm} of the control well × 100. The IC50 values for the extracts and doxorubicin were determined by plotting inhibition (%) against extract and doxorubicin concentrations[25].

Statistical analysis

The results are expressed as the mean ± standard deviation from three independent experiments. Statistical analyses were conducted using SPSS software (version 20.0; IBM, Armonk, NY, USA). Statistically significant differences between the values of the treated and control cells were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test at p-value<0.05.

Results

Total phenolic and flavonoid contents

The TPC and TFC analyses of the extracts from the fruits of *C. excavata* (FCE), roots and leaves of *M. pachycarpa* (RMP and LMP), and twigs and leaves of *U. grandiflora* (TUG and LUG) showed that all the extracts contained varying amounts of phenolic and flavonoid compounds. TUG had the highest TPC (81.06±0.47 µg of gallic acid equivalents/mg of extract), followed by RMP, LUG, LMP, and FCE (68.59±1.41, 50.42±0.94, 42.01±1.24, and 22.21±1.41 µg of gallic acid equivalents/mg of extract, respectively). TUG had the highest TFC (56.48±1.91 µg of catechin equivalents/mg of extract), followed by LUG, LMP, RMP, and FCE (39.45±1.19, 31.09±1.71, 15.31±0.98, 15.16±0.98 µg of catechin equivalents/mg of extract, respectively).

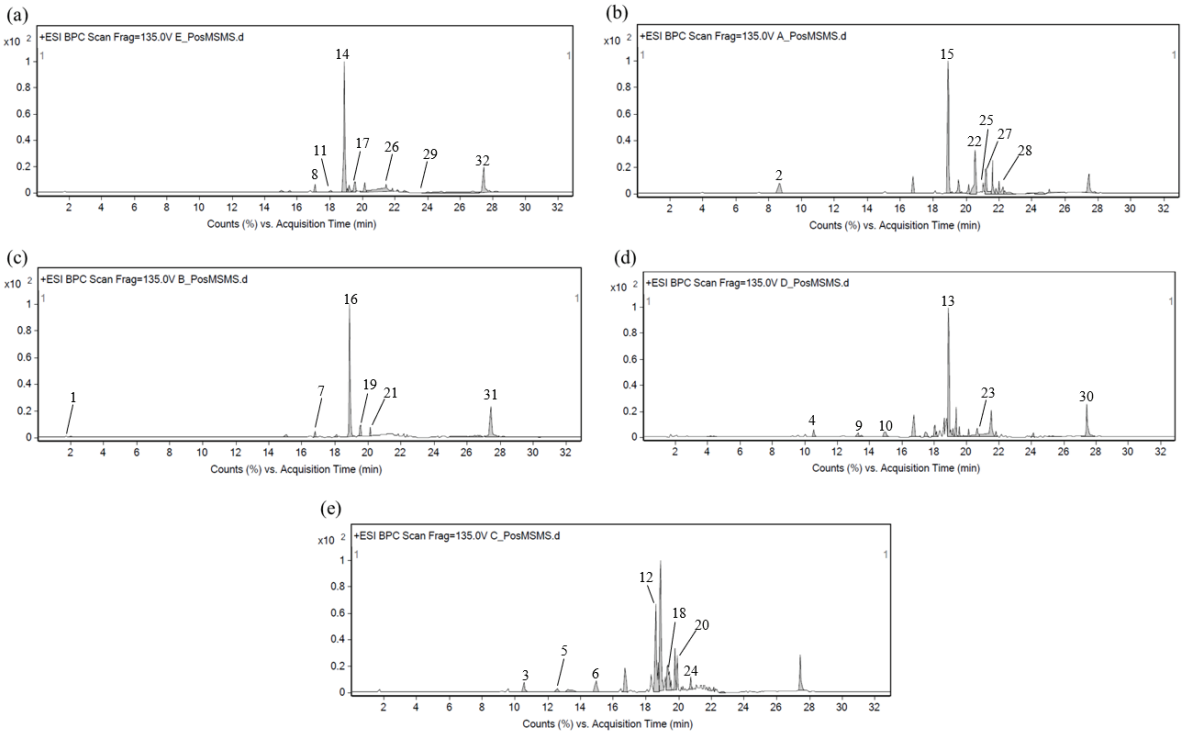
Chemical profile

The chemical profiles of all extracts determined using HPLC-ESI-QTOF-MS were compared with previously published data (ScienceDirect, SciFinder, and Scholar), and chemical constituents scoring higher than 80 were selected for analysis using the PCDL. Chemical formulae were determined using mass errors less than ± 5 ppm for *m/z* verification and mass analysis of the compounds in positive ion mode, as shown in Figure 1 for FCE, RMP, LMP, TUG, and LUG; their known compounds are shown in Table 1. The major constituents identified in FCE were carbazole alkaloids (*e.g.,* girinimbine), xanthones (*e.g.,* lichexanthone), and coumarins (*e.g.,* auraptene). The major constituents identified in RMP and LMP were isoflavonoids (*e.g.,* 6″,6″-dimethyl-5-hydroxy-3′,4′-dimethoxypyrano[2″,3″:7,6] isoflavone), coumarins (*e.g.,* 4-hydroxy-5,6,7-trimethoxy-3-(3′,4′-methylenedioxy) phenylcoumarin), and rotenoids (*e.g.,* usararotenoid C). The major constituents found in TUG and LUG were

alkaloids (*e.g.,* azafluorene), polyoxygenated cyclohexenes (*e.g.,* zeylenol), and flavonoids (*e.g.,* isochamuvaretin, pinostrobin). Since the compounds listed in Table 1 were putative assignments based on library matching, further confirmation with standard compounds or MS/MS tandem mass spectrometry is needed.

Antioxidant and anti-inflammatory activities

Various free-radical-scavenging activities and the MCA and FRAP of FCE, RMP, LMP, TUG, and LUG were determined to study the antioxidant activities of the extracts. TUG, RMP, and LMP exhibited the highest DPPH-radical-scavenging activity with an IC50 value of 1.53±0.06, 1.78±0.04, and 1.88±0.05 µg/mL, respectively. Meanwhile, RMP demonstrated the most potent ABTS- radical-scavenging activity and superoxide-radical-scavenging activity, with IC50 values of 0.15±0.00 and 0.04±0.00 µg/mL, respectively. LUG showed the highest MCA with an IC50 value of 0.08±0.00 µg/mL. Furthermore, LMP exhibited the greatest FRAP, with a value of 32.48±1.12 µmol Fe^{2+} equivalents/mg. However, the positive controls used in the antioxidant assays, including Trolox, catechin, and EDTA, revealed higher DPPH, ABTS, superoxide radical scavenging activities, and MCA, respectively, than the extracts (Table 2). In addition, anti-inflammatory activity tests demonstrated that all extracts inhibited NO production in LPS-stimulated RAW 264.7 cells, in a dose-dependent manner (Figure 2). FCE exhibited the most potent NO inhibitory activity, with an IC50 value of 114.67±7.94µg/mL, whereas indomethacin, the positive control in the anti-inflammatory assay, showed higher NO inhibitory activity than the extracts (Table 3).



LC-MS=liquid chromatography–mass spectrometry, FCE=fruit ectract of *C. excavata*, RMP=root extract of *M. pachycarpa*, LMP=leaf extract of *M. pachycarpa*, TUG=twig extract of *U. grandiflora*, LUG=leaf extract of *U. grandiflora*

Figure 1 LC-MS chromatogram of (a) FCE, (b) RMP, (c) LMP, (d) TUG, and (e) LUG

Table 1 Chemical constituents of FCE, RMP, LMP, TUG, and LUG analyzed by HPLC-ESI-QTOF-MS

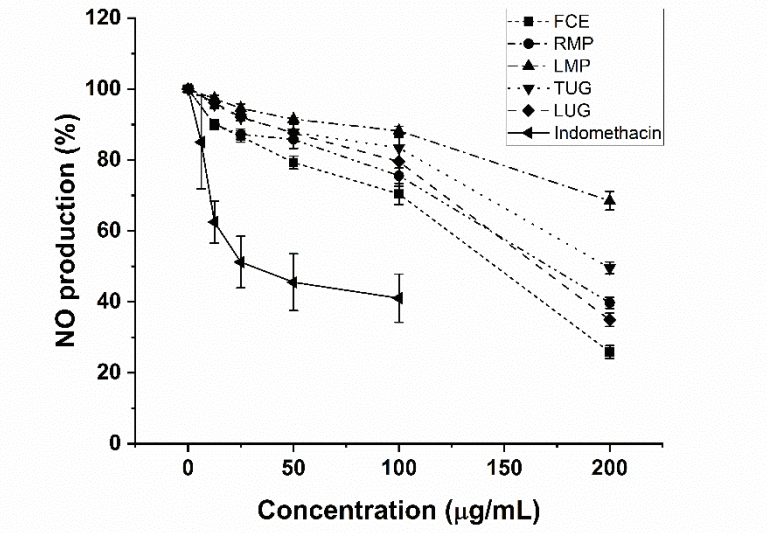
No.	RT (min)[1]	Mass	*m/z* (Expected)[2]	Chemical Formula	Error (ppm)[3]	Identification	FCE	RMP	LMP	TUG	LUG	Area Sum (%)
1	1.985	228.0775	229.0841	$C_{14}H_{12}O_3$	−4.23	desmethoxykanugin			√			0.58
2	8.656	358.067	359.0743	$C_{18}H_{14}O_8$	−4.29	12-dihydrousararotenoid A		√				3.87
3	10.560	280.0953	303.0845	$C_{14}H_{16}O_6$	2.07	cherrevenol G					√	0.80
4	10.570	280.0949	303.0842	$C_{14}H_{16}O_6$	0.88	cherrevenol G				√		3.01
5	12.579	302.0772	303.0846	$C_{16}H_{14}O_6$	−6.08	grandiuvarone A					√	1.39
6	14.952	122.0365	123.0438	$C_7H_6O_2$	−2.24	benzoic acid					√	4.25
7	16.800	328.0939	351.0834	$C_{18}H_{16}O_6$	−2.31	7-hydroxy-2',4',5'-trimethoxyisoflavone			√			0.93
8	17.087	342.1116	365.1003	$C_{19}H_{18}O_6$	3.55	excavatin K	√					9.21
9	17.476	275.0589	298.0482	$C_{17}H_9NO_3$	2.35	liriodenine				√		1.85
10	18.036	265.0722	283.1060	$C_{16}H_{11}NO_3$	−6.48	aristolactam AII				√		4.36
11	18.051	390.1299	391.1373	$C_{20}H_{22}O_8$	−3.98	clauslactone S	√					1.25
12	18.600	384.1216	407.1108	$C_{21}H_{20}O_7$	1.86	zeylenol					√	22.73
13	18.859	167.0744	185.1075	$C_{12}H_9N$	5.25	azafluorene				√		36.97
14	18.874	286.0844	309.0737	$C_{16}H_{14}O_5$	0.87	lichexanthone	√					35.82
15	18.876	256.2399	274.2738	$C_{16}H_{32}O_2$	−1.10	palmitic acid		√				38.72
16	18.891	256.2410	274.2748	$C_{16}H_{32}O_2$	2.91	palmitic acid			√			52.19
17	19.181	199.0637	200.0710	$C_{12}H_9NO_2$	2.11	dictamine	√					2.8
18	19.324	384.1198	407.1092	$C_{21}H_{20}O_7$	−2.76	zeylenol					√	10.43
19	19.553	426.1308	449.1213	$C_{23}H_{22}O_8$	−1.55	12a-hydroxy-a-toxicarol			√			5.16
20	19.788	182.0585	183.0656	$C_9H_{10}O_4$	3.51	3,4-dimethoxybenzoic acid					√	4.84
21	20.150	346.1055	347.1127	$C_{18}H_{18}O_7$	0.69	brandisianin F			√			2.71
22	20.549	380.1257	381.1335	$C_{22}H_{20}O_6$	−0.74	6″,6″-dimethyl-5-hydroxy-3′,4′-dimethoxypyrano[2″,3″:7,6]isoflavone		√				13.47
23	20.634	270.0872	271.0945	$C_{16}H_{14}O_4$	−7.46	pinostrobin				√		9.14
24	20.722	354.1476	377.1368	$C_{21}H_{22}O_5$	2.58	cherrevenol L					√	2.12
25	21.218	366.1107	367.1178	$C_{21}H_{18}O_6$	0.92	6″,6″-dimethyl-5-hydroxy-3′-methoxy,4′-hydroxypyrano[2″,3″:7,6]isoflavone		√				8.25
26	21.434	298.1567	299.1628	$C_{19}H_{22}O_3$	−0.52	auraptene	√					3.42
27	21.591	410.1370	433.1262	$C_{23}H_{22}O_7$	0.99	usararotenoid C		√				5.58
28	22.223	408.1211	409.1284	$C_{23}H_{20}O_7$	0.58	(−)-6a,12a-dehydrotoxicarol		√				4.11
29	23.842	574.4245	597.4092	$C_{35}H_{58}O_6$	1.98	steroidal glucoside	√					1.6
30	27.427	452.1632	453.1660	$C_{29}H_{24}O_5$	1.76	isochamuvaretin				√		10.83
31	27.454	372.0845	390.1188	$C_{19}H_{16}O_8$	0.01	4-hydroxy-5,6,7-trimethoxy-3-(3′,4′-methylenedioxy) phenylcoumarin			√			24.32
32	27.468	263.1309	281.1654	$C_{18}H_{17}NO$	−0.37	girinimbine	√					42.91

[1]RT represent retention time (min), [2] *m/z* show mass-to-charge ratio, [3] Mass error (ppm) show chemical formula was determined using a mass difference tolerance of ±5 ppm, The compound identification was based on PDCL matching.
FCE=fruit ectract of *C. excavata*, RMP=root extract of *M. pachycarpa*, LMP=leaf extract of *M. pachycarpa*, TUG=twig extract of *U. grandiflora*, LUG=leaf extract of *U. grandiflora*, HPLC-ESI-QTOF-MS=high-performance liquid chromatography–electrosprayionization–quadrupole time-of-flight mass spectrometry

Table 2 Antioxidant capacities of FCE, RMP, LMP, TUG, and LUG

Samples	DPPH (IC50; mg/mL)	ABTS (IC50; mg/mL)	Superoxide radicals (IC50; mg/mL)	MCA (IC50; mg/mL)	FRAP (μmol Fe^{2+} equivalents/mg)
FCE	4.32±0.10	2.44±0.06	1.84±0.16	0.48±0.02	14.17±1.36
RMP	1.78±0.04*	0.15±0.00*	0.04±0.00*	1.63±0.08	23.81±0.23
LMP	1.88±0.05*	0.74±0.01	N/A	0.24±0.02	32.48±1.12*
TUG	1.53±0.06*	1.02±0.01	N/A	0.32±0.01	20.28±0.90
LUG	4.78±0.28	3.03±0.08	3.86±0.32	0.08±0.00*	7.65±0.45
Trolox	0.01±0.00	0.05±0.00	–	–	–
Catechin	–	–	0.13±0.02	–	–
EDTA	–	–	–	0.01±0.00	–

*Highest antioxidant capacities among the extracts (p-value<0.05), ±0.00 means S.D. <0.005
ABTS=2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonicacid, DPPH=2,2-diphenyl-1-picrylhydrazyl, MCA=metal-chelating activity, FRAP=ferric-ion-reducing antioxidant power, FCE=fruit ectract of *C. excavata*, RMP=root extract of *M. pachycarpa*, LMP=leaf extract of *M. pachycarpa*, TUG=twig extract of *U. grandiflora*, LUG=leaf extract of *U. grandiflora*, EDTA=ethylenediaminetetraacetic acid



LPS=lipopolysaccharide, FCE=fruit ectract of *C. excavata*, RMP=root extract of *M. pachycarpa*, LMP=leaf extract of *M. pachycarpa*, TUG=twig extract of *U. grandiflora*, LUG=leaf extract of *U. grandiflora*

Figure 2 NO production in RAW 264.7 cells stimulated with 1 μg/mL LPS and treated with various concentration of FCE, RMP, LMP, TUG, LUG and indomethacin for 24 h

Cytotoxicity of the extracts against drug-sensitive and -resistant cancer cells and normal cells

K562 and MCF-7 cells were used as drug-sensitive suspension and adherent cell models, respectively, whereas K562/adr and MCF-7/adr cells were used as drug-resistant suspension and adherent cell models,

Table 3 IC50 values of FCE, RMP, LMP, TUG, LUG, and indomethacin to inhibit NO production in RAW 264.7 cells stimulated with 1 μg/mL LPS and treated with the exracts and indomethacin for 24 h

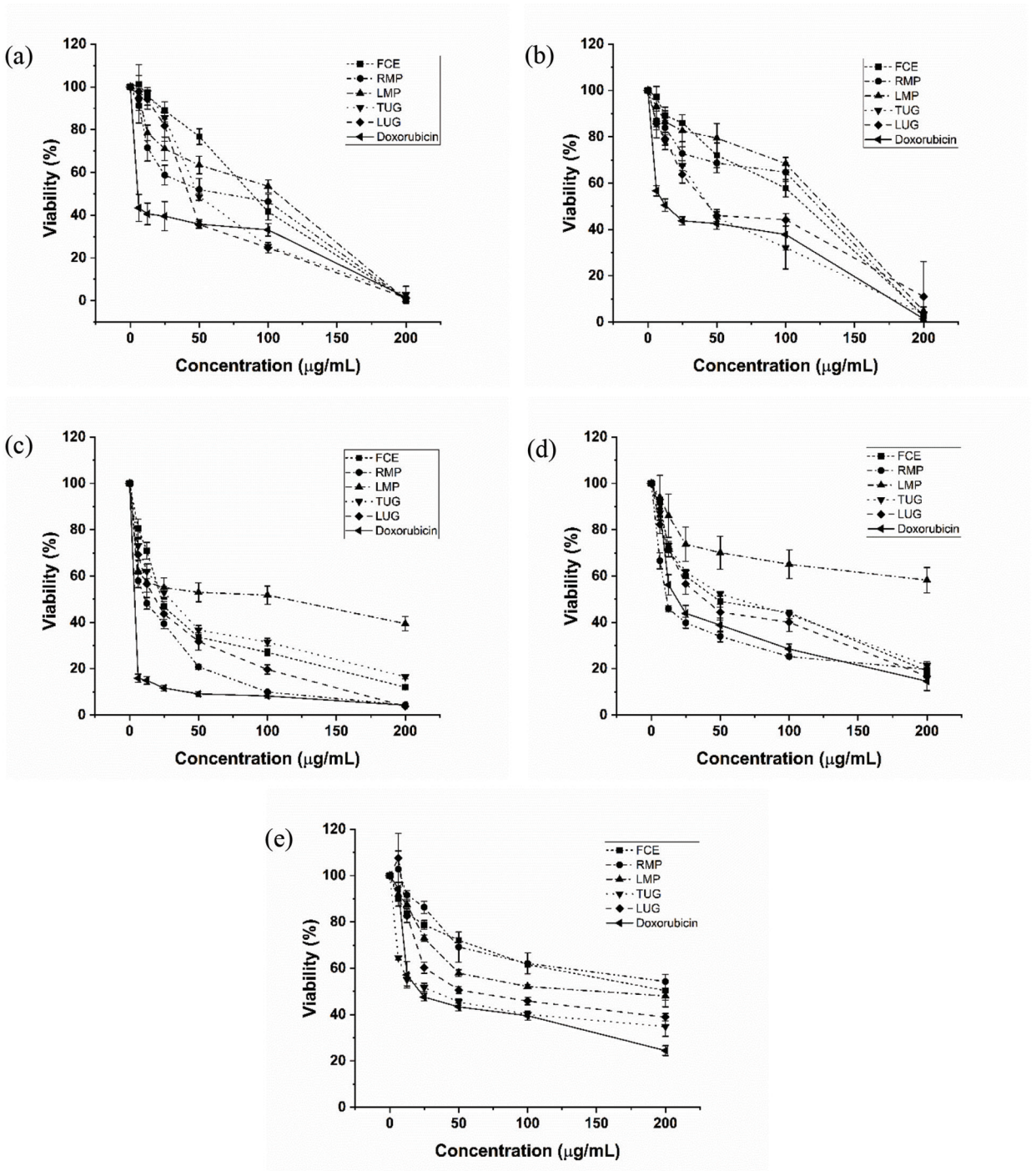
Samples	IC50 (mg/mL)
FCE	114.67±7.94*
RMP	139.75±2.34
LMP	N/A
TUG	160.69±11.67
LUG	139.13±12.96
Indomethacin	11.84±0.75

*Highest anti-inflammatory activity among the extracts (p-value<0.05)
FCE=fruit ectract of *C. excavata*, RMP=root extract of *M. pachycarpa*, LMP=leaf extract of *M. pachycarpa*, TUG=twig extract of *U. grandiflora*, LUG=leaf extract of *U. grandiflora*

respectively. PBMCs were used as the normal cell model. Cytotoxicity tests of the extracts and the standard chemotherapeutic drug doxorubicin were performed in these cells. All extracts exhibited cytotoxicity by reducing the viability of both doxorubicin-sensitive and doxorubicin-resistant erythromyogenic leukemic cells (K562, K562/adr) and breast cancer cells (MCF-7, MCF-7/adr) in a dose-dependent manner (Figure 3, a–d). The viability of normal PBMCs was reduced by all extracts in a dose-dependent manner, as shown in Figure 3e.

The IC50 value was used to compare the cytotoxicity level of each extract and doxorubicin. TUG and LUG exhibited the highest cytotoxicity against both K562 cells, with 58.38±0.62 and 51.74±2.35, respectively, and K562/adr cells, with IC50 values of 55.84±4.60 and 61.66±6.31 μg/mL, respectively. Meanwhile, RMP demonstrated the highest cytotoxicity against MCF-7 and MCF-7/adr cells, with IC50 values of 22.50±1.60 and 28.83±1.93 μg/mL, respectively. Whereas doxorubicin, serving as the positive control in the cytotoxicity assay, revealed greater cytotoxicity against all the tested cancer cells. Furthermore, FCE and RMP exhibited the least cytotoxicity to PBMCs, with IC50 values greater than 200 μg/mL. However, LMP and LUG were less cytotoxic to PBMCs than doxorubicin (Table 4).

The resistance factor (RF) was calculated by dividing the IC50 value of the extract in doxorubicin-resistant cancer cells by the IC50 value of the extract in doxorubicin-sensitive cancer cells. An RF value not exceeding 1 indicated that the extract could overcome the drug resistance of cancer cells. Conversely, an RF value greater than 1 indicated that the extract could not overcome drug resistance in cancer cells. The analysis revealed that TUG could overcome the drug resistance of erythroleukemic cells with an RF value of 0.96. Nevertheless, TUG also exhibited cytotoxicity to normal PBMCs with an IC50 lower than the cancer cells. Thus, it might be difficult to develop TUG as an alternative drug in the future, and it might be limited to TUG use. In contrast, FCE, RMP, LMP, and LUG did not overcome drug resistance in erythromyelogenous leukemic cells. Additionally, none of the 5 extracts overcame drug resistance in breast cancer cells. However, all extracts exhibited lower RF values than doxorubicin for erythromyelogenous leukemic and breast cancer cells (Table 5). This suggested that all extracts retained greater anticancer efficacy against drug-resistant cancer cells compared to doxorubicin.



FCE=fruit ectract of *C. excavata*, RMP=root extract of *M. pachycarpa*, LMP=leaf extract of *M. pachycarpa*, TUG=twig extract of *U. grandiflora*, LUG=leaf extract of *U. grandiflora*

Figure 3 Cell viability of (a) K562, (b) K562/adr, (c) MCF-7, (d) MCF-7/adr, and (e) PBMCs treated with various concentration of FCE, RMP, LMP, TUG, LUG, and doxorubicin for 72 h

Table 4 IC50 values of FCE, RMP, LMP, TUG, LUG, and doxorubicin in K562, K562/adr, MCF-7, MCF-7/adr, and PBMCs treated with the extracts and doxorubicin for 72 h

Samples	IC50 (mg/mL)				
	K562	K562/adr	MCF-7	MCF-7/adr	PBMC
FCE	91.12±5.84	116.13±12.12	38.50±2.46	59.67±1.44	>200[#]
RMP	66.26±7.78	114.48±14.55	22.50±1.60[*]	28.83±1.93[*]	>200[#]
LMP	89.49±8.74	132.02±6.63	67.59±15.96	>200	84.84±3.97
TUG	58.38±0.62[*]	55.84±4.60[*]	40.24±3.45	62.65±1.84	44.22±1.86
LUG	51.74±2.35[*]	61.66±6.31[*]	31.22±4.65	53.49±7.35	63.12±2.84
Doxorubicin	<6.25	40.10±6.22	<6.25	38.66±3.94	46.93±0.51

[*]Highest cytotoxicity among the extracts (p-value<0.05), [#] Lowest cytotoxicity (p-value<0.05)
FCE=fruit ectract of *C. excavata*, RMP=root extract of *M. pachycarpa*, LMP=leaf extract of *M. pachycarpa*, TUG=twig extract of *U. grandiflora*, LUG=leaf extract of *U. grandiflora*

Table 5 Resistance factor (RF) of FCE, RMP, LMP, TUG, LUG, and doxorubicin in erythromyelogenous leukemic and breast cancer cells

Samples	RF	
	Erythromyelogenous leukemic cells	Breast cancer cells
FCE	1.27	1.55
RMP	1.73	1.28
LMP	1.48	>2.96
TUG	0.96	1.56
LUG	1.19	1.71
Doxorubicin	>6.42	>6.19

FCE=fruit ectract of *C. excavata*, RMP=root extract of *M. pachycarpa*, LMP=leaf extract of *M. pachycarpa*, TUG=twig extract of *U. grandiflora*, LUG=leaf extract of *U. grandiflora*

Discussion

Phytochemicals, particularly the compounds in phenolic and flavonoid groups, exhibit a range of biological activities and health benefits in humans[2,3]. The TPC and TFC of extracts from the fruits of *C. excavata,* roots and leaves of *M. pachycarpa*, and the twigs and leaves of *U. grandiflora* were measured in this study. All extracts contained varying amounts of phenolic and flavonoid compounds. Chemical profile analysis of the extracts revealed biologically active compounds in the polyphenol and flavonoid groups, which varied in composition. FCE contained carbazole alkaloids, xanthones, and coumarins. RMP and LMP contained isoflavonoids, coumarins, and rotenoids. TUG and LUG contained alkaloids, polyoxygenated cyclohexenes, and flavonoids. These findings align with those of previous studies showing that fruit extracts of *C. excavata* contain compounds such as coumarins and carbazole alkaloids[6], and its root and stem bark extracts contain limonoids, coumarins, and carbazole alkaloids[5]. The root and leaf extracts of *M. pachycarpa* contain rotenoids and isoflavonoids[10,26,27],

and the twig and leaf extracts of *U. grandiflora* contain polyoxygenated cyclohexenes and flavonoids[11-13].

This study demonstrated the antioxidant activities of *C. excavata*, *M. pachycarpa*, and *U. grandiflora*, including DPPH- and ABTS-radical-scavenging activities and FRAP, as reported in several previous studies. Thant et al. reported that coumarins and carbazole alkaloids isolated from the roots of *C. excavata* exhibit DPPH-radical-scavenging activity[7]. Tanruean et al. reported that the methanolic leaf extract of *C. excavata* exhibited ABTS-radical-scavenging activity and FRAP[28]. The methanolic leaf extract of *M. pachycarpa* revealed ABTS-radical-scavenging activity and FRAP[29], whereas stem bark extracts prepared using chloroform and ethanol showed DPPH-radical-scavenging activity[30]. In addition, alkaloids isolated from the twigs of *U. grandiflora* exhibited DPPH-radical-scavenging activity[13].

The anti-inflammatory activity of the extracts was determined based on the inhibition of NO production in LPS-induced RAW 264.7 cells. NO is a proinflammatory mediator generated during inflammation. In RAW 264.7 cells, inflammation accompanied by NO production was triggered by LPS. The fruit extract of *C. excavata*, root extract of *M. pachycarpa*, and twig extract of *U. grandiflora* exhibited anti-inflammatory activities by inhibiting NO production in LPS-induced RAW 264.7 cells. Previous studies have shown that the methanolic leaf extract of *C. excavata* inhibits NO production in LPS-activated mouse macrophages (J774A.1)[31]. The ethanolic extract of *M. pachycarpa* has been shown to inhibit NO production in LPS-induced RAW 264.7 cells[8,32]. Polyoxygenated cyclohexene and zeylenol isolated from the stems of *U. grandiflora* exhibit anti-inflammatory activity in a rat model[12].

The present observation of the cytotoxicity of all the extracts against K562, K562/adr, MCF-7, and MCF-7/adr cancer cells demonstrated that each extract exhibited different degrees of cytotoxicity against these cancer cells. This observation is consistent with those of previous studies on the cytotoxicity of bioactive compounds isolated from *C. excavata*, *M. pachycarpa*, and *U. grandiflora* in various types of cancer cells. Coumarins isolated from *C. excavata* fruits exhibit weak cytotoxicity against human cancer cells, such as lung cancer (A549), colorectal cancer (SW480), and K562 cells[6], whereas limonoids and coumarins isolated from the root and stem bark of *C. excavata* exhibit cytotoxicity against promyelocytic leukemia (HL-60), cervical cancer (HeLa), colon cancer (HT-29), and MCF-7 cells[5]. Chalcones isolated from the seeds of *M. pachycarpa* are also cytotoxic against human hepatocellular carcinoma (HepG2), renal adenocarcinoma (786-O), ovarian adenocarcinoma (A2780), A549, HeLa, and K562 cells[33], whereas rotenoids and isoflavonoids isolated from the roots and leaves of *M. pachycarpa* are cytotoxic against A549, SW480, and K562 cells[11]. Other reports have shown that polyoxygenated cyclohexenes isolated from the stems and leaves of *U. grandiflora* are cytotoxic against breast cancer (MDA-MB231), HepG2, K-562, and HeLa cells[12,34].

To investigate the potential of extracts for overcoming multidrug resistance in cancer cells, doxorubicin-resistant erythromyelogenous leukemia (K562/adr) and breast cancer cells (MCF-7/adr) were used as models and exhibited higher IC50 values of doxorubicin than those in doxorubicin-sensitive erythromyelogenous leukemia (K562) and breast cancer cells (MCF-7). This can be explained by the mechanisms of multidrug resistance in cancer cells, such as the expression of ATP-dependent efflux pumps (ATP-dependent drug transporters), the inhibition of apoptosis by altering the function of cell cycle checkpoints, and the activation of gene expression for cell survival[35]. This study demonstrated that the twig extracts of *U. grandiflora* could overcome multidrug resistance in erythromyelogenous leukemic cells, which is consistent with the findings of previous studies on plant-derived bioactive compounds

against multidrug resistance in erythromyelogenous leukemic cells[16-18]. This indicated that the extracts might inhibit such mechanisms of multidrug resistance in cancer cells.

The evaluation of toxicity and the adverse effects of the extracts showed that fruit extracts of *C. excavata* and root extracts of *M. pachycarpa* possess very low cytotoxicity to PBMCs, which is consistent with other studies showing very low toxicity of alkaloids, flavonoids, and polyphenols from plant extracts against normal white blood cells, PBMCs, and other normal cell models[18-20]. This observation can be attributed to the low toxicity and minimal adverse effects of the extracts on human health.

Conclusion

This study demonstrated that all five types of extracts tested contained varying amounts of phenolic and flavonoid compounds. The TUG test showed the highest TPC and TFC. Chemical profile analysis revealed bioactive compounds, such as carbazole alkaloids, xanthones, and coumarins in FCE; isoflavonoids, coumarins, and rotenoids in RMP and LMP; and alkaloids, polyoxygenated cyclohexenes, and flavonoids in TUG and LUG. In terms of antioxidant activity, RMP had the highest ABTS-radical-scavenging activity, LUG showed the highest MCA, and LMP had the greatest ferric-ion-reducing power. The determination of anti-inflammatory activity in LPS-stimulated RAW 264.7 cells revealed that FCE had the most potent NO inhibitory activity. The cytotoxicity of the extracts was determined in K562, K562/adr, MCF-7, and MCF-7/adr cancer cells, normal cells, and PBMCs. The results indicated that TUG and LUG exhibited the highest cytotoxicity against K562 and K562/adr cells, and RMP exhibited the highest cytotoxicity against MCF-7 and MCF-7/adr cells. When calculating the RF, only TUG could overcome drug resistance in the erythromyelogenous leukemic cells. FCE and RMP exhibited the lowest toxicity in PBMCs. Notably, the extracts showed low cytotoxicity towards peripheral PBMCs, suggesting their potential safety and minimal adverse effects on normal cells.

The findings of this study underscore the potential of C. excavata, M. pachycarpa, and U. grandiflora extracts as sources of bioactive compounds with antioxidant, anti-inflammatory, and cytotoxic activities in both drug-sensitive and drug-resistant suspension, and in adherent cancer cells with low toxicity. These findings support future exploration for the development of dietary supplements and potential therapeutic agents, particularly for combating drug-resistant cancers. Moreover, they offer initial insights into the antioxidant, anti-inflammatory, and anticancer activities of the crude extracts from these plants against both drug-sensitive and drug-resistant cancer cells. However, further studies are required to isolate the pure bioactive compounds and examine their pharmacological effects. In addition, research on the mechanisms underlying their anti-inflammatory and anticancer effects is necessary. The pharmacological effects of these extracts should be explored in vivo and clinically.

Acknowledgement

The authors thank Associate Professor Sawitree Chiampanichayakul, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, for providing the doxorubicin-sensitive and -resistant erythromyelogenous leukemic cell lines. The authors also thank Professor Surat Laphookhieo, the Natural Products Research Laboratory, School of Science, Mae Fah Luang University, for providing the plant materials.

Funding sources

The authors are grateful for the research funding provided by the Fundamental Fund, fiscal year 2023 (Grant No. 662A05030), of the National Science, Research and Innovation Fund (NSRF), and Mae Fah Luang University.

Conflict of interest

We declare no conflicts of interest.

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