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RESEARCH ARTICLE

Therapeutic Potential of *Garcinia Forbesii* King Extracts: Antioxidant Effects and Bioassay-Based Evaluation in PANC-1 and HT29-Derived EV Reporter Cells

Asyiqin Zulkiflee[1,2], Norhayati Ahmad[3,4], Peng Du[5], Masayoshi Arai[2,5] and Hartini Mohd Yasin[1,2,*]

[1]*Chemical Sciences, Faculty of Science, Universiti Brunei Darussalam, Gadong BE1410, Brunei Darussalam*
[2]*The University of Osaka ASEAN Campus Brunei Darussalam, Bandar Seri Begawan BA1111, Brunei Darussalam*
[3]*Institute for Biodiversity and Environmental Research, Universiti Brunei Darussalam, Gadong BE1410, Brunei Darussalam*
[4]*Environmental and Life Sciences, Faculty of Science, Universiti Brunei Darussalam, Gadong BE1410, Brunei Darussalam*
[5]*Graduate School of Pharmaceutical Sciences, The University of Osaka, Osaka 5650871, Japan*

([*]Corresponding author's e-mail: hartini.yasin@ubd.edu.bn)

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Abstract

Garcinia forbesii King (*G. forbesii*) is an indigenous plant of Brunei Darussalam, traditionally used for medicinal purposes. This study evaluated the phytochemical composition, antioxidant activity, and anticancer potential of the fruit flesh and peel prepared via ultrasonication-assisted maceration using *n*-hexane, 80% EtOH, and water as extraction solvents. It was found that 80% EtOH was the most effective solvent for extracting bioactive compounds from *G. forbesii*, yielding a maximum of 63.8%. Terpenoids, saponins, and tannins were detected in all extracts from the flesh and peel. The *n*-hexane peel extract had the highest TPC (27.31 ± 0.82 mg GAE/g DE), while the 80% EtOH peel extract had the highest TFC (5.54 ± 0.50 mg QE/g DE). All extracts exhibited potent antioxidant activities in DPPH, FRAP, and ABTS assays, with the *n*-hexane peel extract showing the highest activity (DPPH IC_{50} = 289.4 mg/L, ABTS IC_{50} = 125.0 mg/L, FRAP = 57.0 ± 0.9 mg TE/g DE). Notably, the *n*-hexane peel extract selectively inhibited PANC-1 cells under glucose-starved conditions, showing the smallest AUC value (0.37), and the 80% EtOH peel extract selectively influenced extracellular vesicle secretion without affecting the cell viability of HT29/CD63-Nluc cells. These findings support *G. forbesii* extracts as a promising potential source of antioxidant and anticancer properties, providing scientific evidence for its traditional medicinal use.

Keywords: *Garcinia forbesii* King, Antioxidant, Sequential extractions, PANC-1, HT29/CD63-Nluc

Introduction

Cancer remains one of the leading causes of death worldwide. According to the World Health Organization, an estimated of 19.3 million new cancer cases and 10 million cancer-related deaths occurred in 2021 [1]. In Brunei Darussalam, about 795 new cancer cases were reported among citizens and residents in 2023, an increase from 743 cases in the previous year, according to the Brunei Darussalam Cancer Registry [2].

In the same year, 351 deaths were attributed to cancer, highlighting growing concern over the rising incidence within the country [2]. The importance of discovering and developing new treatments is highlighted by the growing prevalence of cancer, as well as the drawbacks, efficacy, and high toxicity of conventional chemotherapy [3,4]. Human diseases, particularly cancer and infectious diseases, have long

been treated and prevented with natural products derived from plants, animals, and microorganisms [3-5]. A significant proportion of currently used anticancer drugs originates from plant-derived natural products. For instance, the *Garcinia* species is a well-known source of naturally occurring secondary metabolites with therapeutic potential in treating numerous diseases, including cancer [6-8].

Garcinia species are mostly edible and belong to the *Clusiaceae* family [9,10]. These species typically grow in moist, lowland tropical forests found in Southeast Asia and Madagascar [11]. Furthermore, these types of species are usually distinguished by the presence of a compound called xanthone, which is a naturally occurring secondary metabolite and can be used to treat numerous diseases [12,13]. Therefore, xanthones present a promising pharmacology for novel medications due to their strong bioactivities [14]. Moreover, *Garcinia* has been used as a form of traditional medicine for a long time [9,15,16]. For instance, one of the widely recognized species is *Garcinia mangostana*. The pericarp of *Garcinia mangostana* is commonly used by Southeast Asians for the treatment of skin infections and wounds [15]. Moreover, in Ayurverdic medicine, it is used to treat inflammation and diarrhea [15]. Another example, the bark of *Garcinia cowa* was used as an antipyretic agent, and the fruit and leaves were both used for indigestion and as an expectorant [16]. These traditional uses are supported by reports of bioactive components contributing to antioxidant and therapeutic effects [6].

Recently, *Garcinia forbesii* King (*G. forbesii*), also known as 'asam aur-aur', is an endemic plant found in Brunei Darussalam. This plant can grow up to 18 metres tall in a hot, wet, and humid climate [9,11]. Moreover, the tree, leaves, flowers, and fruit of *G. forbesii* are shown in **Figure 1**. In folklore medicine, *G. forbesii* has been traditionally used to treat diabetes and malaria in the eastern part of Indonesia [17]. Furthermore, the rind has been used for traditional medicinal and culinary purposes for a long time. These include helping women recover from childbirth, treating minor stomach ailments, and coughs [18]. However, its pharmacological potential remains less extensively studied compared to other *Garcinia* species such as *Garcinia mangostana*. Previous studies reported that different parts of *G. forbesii*, extracted using solvents with different polarities, exhibit varying antioxidant and biological activities.

For instance, J. Wairata et al. extracted the bark of *G. forbesii* using *n*-hexane, dichloromethane (DCM), ethyl acetate (EtOAc), and methanol. It is reported that the *n*-hexane extract of the bark showed potent antioxidant activity with the lowest IC_{50} of 8.12 ± 0.02 ppm compared to all extracts [17]. In another study, Larasati *et al*. evaluated the antimicrobial activity of *G. forbesii* leaves using the diffusion agar method and showed potential antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* [19]. In a similar study, Joharman *et al*. [6] macerated the leaves, stem bark, wood, and pericarps using ethanol (EtOH) as a solvent and investigated for anticancer activity on breast cancer (MCF-7) and liver cancer (HepG2) cell lines [6]. The ethanolic extracts demonstrated cytotoxic activity against MCF-7 and HepG2 cell lines, with IC_{50} values of 60.11 µg/mL and 47.10 µg/mL, respectively [6]. Wairata *et al*. [17] carried out *in vitro* anti-diabetic assays using α-glucosidase and α-amylase inhibition tests. In the α-glucosidase assay, DCM and EtOAc extracts showed the lowest IC_{50} values of 35.13 ± 2.01 µg/mL and 24.88 ± 1.27 µg/mL, respectively [17]. The DCM extract also demonstrated the highest inhibitory activity against α-amylase, with an IC_{50} value of 4.83 ± 0.20 µg/mL [17].

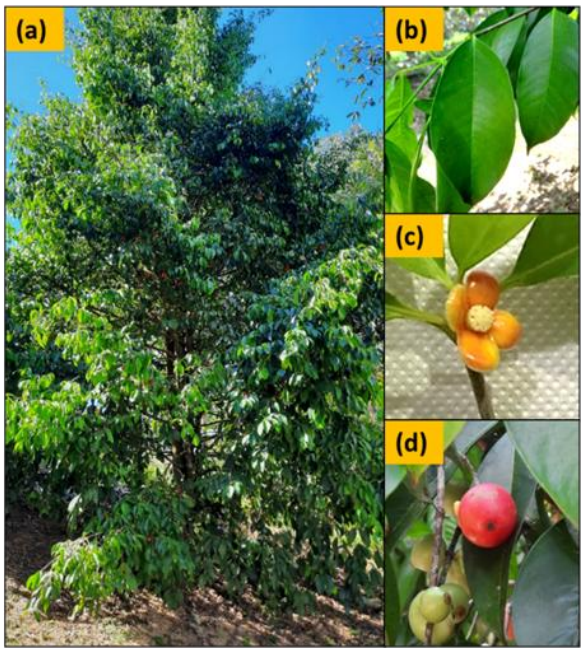


Figure 1 The (a) tree, (b) leaves, (c) flower, and (d) fruits of *G. forbesii*. This tropical species belongs to the *Clusiaceae* family and is traditionally used in folk medicine. The various morphological parts shown are important for botanical identification and for selecting appropriate plant material for phytochemical and pharmacological studies.

Despite these findings, the anticancer potential of *G. forbesii* remains underexplored, particularly in clinically relevant models such as pancreatic cancer (PANC-1) anti-austerity assays and extracellular vesicle-based systems that evaluate tumour progression and intercellular communication. Anti-austerity approaches have garnered significant interest as a means of selectively targeting pancreatic cancer cells that thrive under nutrient-deprived tumour microenvironments [20,21]. Furthermore, the inhibition of tumour-derived extracellular vesicles may suppress cancer progression by disrupting intercellular communication involved in metastasis, angiogenesis, and therapeutic resistance [22-24].

Therefore, in this study, the flesh and peel of *G. forbesii* were sequentially extracted using ultrasonication-assisted maceration with *n*-hexane, 80% (v/v) ethanol (80% EtOH), and water as solvents. To the best of the author's knowledge, this research has not previously reported the anti-austerity activity of the flesh and peel of *G. forbesii* using pancreatic (PANC-1) cells and a human colorectal cancer line engineered to express CD63 fused with a Nanoluc (Nluc) luciferase reporter (HT-29/CD63-Nluc) for assessing cell viability and extracellular vesicle (EV) secretion. Additionally, the percentage yield, qualitative phytochemical screenings, total phenolic content (TPC), and flavonoid content (TFC) were examined. Antioxidant assessments, such as the 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP), and 2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonic acid) (ABTS) assays were also conducted. Furthermore, this study demonstrates the potential *in vitro* anti-austerity activity of the flesh and peel extracts of *G. forbesii* in PANC-1 cells, while HT-29/CD63-Nluc cells were used to evaluate their cytotoxicity and ability to inhibit EV secretion.

Materials and methods

Chemicals used

For the sequential extraction, the chemicals used were *n*-hexane acquired from Merck, Germany and ethanol was sourced from HmbG chemicals, Malaysia. The chemicals used for total phenolic and flavonoid contents, phytochemical screening, and antioxidant activities screening include aluminium chloride hexahydrate, sodium carbonate, hydrochloric acid, sulfuric acid, chloroform, ammonia, sodium hydroxide, and Folin-Ciocalteu's phenol reagent were obtained from Merck, Germany. The glacial acetic acid was obtained from BDH, UK. 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-

carboxylic acid (Trolox), ascorbic acid, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), quercetin, gallic acid, potassium acetate, sodium acetate, ferric chloride hexahydrate, and Dragendorff's reagent were acquired from Sigma Aldrich, Germany. All of the chemicals were used without any further treatment. Throughout the experiment, double-distilled water was used, which was purified using an Aquatron system from England.

For the anticancer assay, pancreatic cancer cell line was obtained from the Riken BioResource Research Center (BRC), Japan. HT-29/CD63-Nluc, a human colon cancer cell line that stably expresses Nanoluc-fused CD63, was previously generated [25] and kindly provided by Oneyama *et al*. [25] The Dulbecco's Modified Eagle Medium (DMEM) high glucose was sourced from Nacalai Tesque, Inc., Kyoto, Japan. The culture media, containing 10% fetal bovine serum (FBS) and 50 µg/mL kanamycin, were acquired from Equitech-Bio Inc. (Kerrville, TX, USA) and Fujifilm Wako Pure Chemical Corporation, (Tokyo, Japan), respectively. PANC-1 cells were cultured in glucose-free DMEM (Nacalai Tesque, Inc., Kyoto, Japan) containing 10% dialyzed FBS (Equitech-Bio Inc., Kerrville, TX, USA) and 50 µg/mL kanamycin to induce cell adaptation to nutrient starvation. The McCoy's 5A medium was purchased from the Gibco Thermo Fisher Scientific Inc. (Waltham, MA, USA). The luciferase buffer and substrate were obtained from the Nano-Glo Luciferase Assay System (Promega, Madison, WI, USA) to measure luminescence. To determine the cell viability, the Water-Soluble Tetrazolium 8 (WST-8) assay was acquired from Nacalai Tesque, Inc. (Kyoto, Japan).

Plant preparation

The fruits of *G. forbesii* were collected from Kg. Jerudong (4°55'23.9" N 114°50'58.3" E). The fruits were initially washed with water to remove any dirt before being allowed to air-dry at room temperature. The fruits were cut in half to extract the flesh and peel. The flesh and peel were then dried at 60 °C in a dehydrator until their mass remained constant. After drying, the samples were ground and sieved through a 300-micron sieve. The brown (flesh) and red-orange (peel) powders were ground to a size of less than 300 microns. These powders were stored in vials at 4 °C until further use.

Extraction method

A sample-to-solvent ratio of 1:20 (w/v) was used for all extractions to ensure consistency. The samples were ultrasonicated in *n*-hexane for 2 h, and the resulting solution was filtered using filter paper (Whatman No. 1) via gravitational filtration. The collected residue was re-extracted twice with fresh *n*-hexane under the same conditions (three successive extraction cycles in total). All collected extracts were combined and concentrated using a rotary evaporator (Buchi R-114, Switzerland) under reduced pressure to obtain the *n*-hexane crude extract. Sequential extraction of the remaining residues was then performed using solvents in order of increasing polarity under identical extraction conditions. Other solvents used in the extraction were 80% EtOH and water. All extraction procedures were performed under controlled and standardised conditions with fixed solvent ratio and extraction time to ensure reproducibility. The percentage yield of each crude extract was recorded.

Qualitative phytochemical screening

The phytochemical screening was carried out as outlined by Harborne *et al*. [26], Edeoga *et al*. [27], and Aiyegoro and Okoh *et al*. [28] with slight modifications. These sample extracts were qualitatively tested for alkaloids, terpenoids, saponins, tannins, anthraquinone glycosides, and glycosides. To prepare a stock solution of each crude extract, 100 mg of extract was dissolved in 10 mL of its respective solvent. A "+" symbol indicates the presence of these phytochemicals, while a "–" symbol indicates their absence.

Alkaloid

Alkaloids were detected using Dragendorff's reagent, following the method of Kancherla *et al*. [29]. The formation of a red-orange precipitate indicates a positive result.

Saponin

The persistent formation of a foam layer after vigorous shaking showed the presence of saponins.

Tannins

The presence of tannins can be confirmed by the ferric chloride test, where the formation of a blue-green colouration or precipitate indicates a positive result.

Terpenoid

Using the Salkowski test, the appearance of reddish-brown colouration at the interface indicates the presence of terpenoids.

Glycoside

Using the Keller-Killiani test, when glycosides are present, the junction of the two layers forms a reddish-brown ring [30].

Anthraquinone glycoside

Following Borntrager's test protocol, as reported by Nortjie *et al*. [31], the formation of a rose pink to red colouration indicated the presence of anthraquinone glycosides.

Total phenolic content

The quantitative phenolic content (TPC) was evaluated using the Folin-Ciocalteu method, following the procedure described by Djordjevic *et al*. [32], Hanifa *et al*. [33], and Wairata *et al*. [17] with slight modifications. Gallic acid was used as a calibration standard. From the stock solution (500 ppm), different concentrations of gallic acid (0 - 200 ppm) diluted in 80% EtOH were prepared. Crude extracts (500 ppm) were mixed with 2.5 mL of 10% (v/v) Folin-Ciocalteu solution and left to stand for 5 min. After incubation, 2 mL of 7.5% (w/v) sodium carbonate solution was added to the mixture. The mixture was further incubated for 60 min at 20 - 25 °C. After that, the mixture (270 µL) was pipetted into the 96-well microtiter plate. A microplate reader (BIOBASE ELISA microplate reader, BK-EL10C, China) was used to measure absorbance at 765 nm. The results are expressed as mg gallic acid equivalent (GAE) per gram of crude extract.

Total flavonoid content

According to the method described in the reported studies [17,34,35], the total flavonoid content (TFC) was determined using the aluminium chloride colorimetric method, with some modifications. Quercetin standards were prepared in 80% EtOH, and the extracts were prepared at 500 ppm in their respective solvents. Subsequently, 0.5 mL of the extracts or quercetin was mixed with 0.1 mL of 10% (w/v) aluminium chloride, 0.1 mL of potassium acetate (pH 7.5), and 1.5 mL of its mother solvent. The mixture was incubated for 60 min at 20 - 25 °C. At the end of the incubation period, 270 µL of the mixture was pipetted into the 96-well microplate. The absorbance was measured at 415 nm using a microplate reader. TFC was evaluated from the quercetin calibration graph and expressed as mg Quercetin equivalent (QE) per gram of crude extract.

Antioxidant assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH radical scavenging activity of the crude extracts was conducted in accordance with the method outlined by reported studies, with slight changes [12,14,36]. Gallic acid (0 - 10 ppm) was used as the calibration standard, and samples were prepared in their respective solvents. Briefly, 90 µL of sample or gallic acid, followed by 180 µL of DPPH solution, were added to a 96-well microplate. The mixture was incubated for 30 min at a temperature of 20 - 25 °C. A microplate reader was used to measure absorbance at 514 nm. The antioxidant activity was calculated and expressed as a percentage of inhibition using the formula as shown in Eq. (1);

$$\%Inhibition = \frac{Abs\ (blank) - Abs\ (sample)}{Abs\ (blank)} \times 100\% \quad (1)$$

Antioxidant activity was also expressed as the inhibitory concentration (IC_{50}) using Eq. (2), representing the concentration required to inhibit 50% of free radicals.

$$IC_{50} = \frac{50 - a}{b} \quad (2)$$

Ferric Reducing Antioxidant Power (FRAP) assay

FRAP assay was conducted according to the procedure reported by previous studies with minor modifications [12,37,38]. Trolox was employed as the calibration standard, with concentrations ranging from 0 - 500 ppm. The FRAP reagent was prepared by mixing 10 mM TPTZ, 20 mM $FeCl_3 \cdot 6H_2O$, and 300 mM sodium acetate buffer (pH 3.6) in a 1:1:10 ratio. Each extract was prepared at a concentration of 500 ppm in its respective solvent. In a vial, 100 µL of the extract solution, 900 µL of double-distilled water, and 2 mL of FRAP reagent were added, and the mixture was

incubated at 37 °C for 30 min. Subsequently, 270 µL of the mixture was transferred into the 96-well microplate. The absorbance was measured at 593 nm using a microplate reader. The results were expressed as mg Trolox equivalent (TE) per gram of extract.

2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS) assay

The ABTS assay is one of the methods to determine the antioxidant activities of the extracts, following the method outlined by Roberta *et al*. [39], Thaipong *et al*. [40], and Rajurkar [41]. Firstly, 7 mM ABTS dissolved in 80% EtOH and 2.45 mM potassium persulfate solution in a 1:1 ratio were mixed to prepare the ABTS reagent. The ABTS reagent was incubated in the dark for 12 - 16 h. After incubation, 10 mL of the ABTS reagent was taken and further diluted with 80% EtOH to give an absorbance of 0.70 ± 0.02. Trolox (0 - 20 ppm) was used as the standard, while the extracts were diluted at concentrations ranging from 0 - 5,000 ppm in their respective solvents. Approximately 90 µL of the prepared extracts or standard solution and 180 µL of ABTS reagent were dispensed into the 96-well microplate and incubated for 30 min. Absorbance was measured at 765 nm using a microplate reader. Results were expressed as a percentage of inhibition and IC_{50} using Eqs. (1) and (2), respectively.

Evaluation of anti-austerity activity against PANC-1 cells

PANC-1 cells were evaluated using a Water-Soluble Tetrazolium 8 (WST-8) assay according to the procedure described by Sirimangkalakitti *et al*. [3], Zaher *et al*. [42], and Tang *et al*. [43]. PANC-1 cells were seeded in a 96-well culture plate at a high density of 10,000 cells/100 µL/well in DMEM high glucose medium and incubated overnight in a humidified atmosphere with 5% CO_2 at 37 °C. The medium was then removed, washed twice with phosphate-buffered saline (PBS, 9.6 g/L), and replaced with either DMEM high glucose (+ve glu) or DMEM no glucose (–ve glu) to induce cell adaptation to nutrient starvation. After 12 h of incubation, crude extracts dissolved in DMSO were added at concentrations ranging from 10 to 100 µg/mL, followed by an additional 12 h of incubation. DMSO served as a negative control, and Antimycin A as a positive control. Cell viability was finally assessed by 4 h incubation with 5% and 7.5% WST-8 for +ve glu and –ve glu conditions, respectively. Absorbance was measured at 450 nm using a microplate reader (BIO-RAD iMark microplate reader, US) to determine the anticancer activity and selectivity of the crude extracts.

Evaluation of cell viability and luciferase activity against HT-29/CD63-Nluc cells

Following the methods described by the reported studies [3,42,44]. The HT-29/CD63-Nluc, a human colon cancer cell line that expresses Nanoluc-fused CD63, was seeded in a 96-well culture plate at a density of 2,000 cells/100 µL/well in McCoy 5A medium and incubated overnight in a humidified atmosphere with 5% CO_2 at 37 °C. The medium was then replaced with fresh medium, and the samples were added. Subsequently, the plate was incubated for 48 h in the same conditions. The effects of the extracts on the cytotoxicity and luciferase activity of HT-29/CD63-Nluc cells were evaluated using 2 complementary assays, a cell viability assay and a luciferase-based luminescence assay. The crude extracts were prepared by dissolving in DMSO in concentrations of 10 - 100 µg/mL. To measure luminescence, 50 µL of the supernatant was transferred to a white opaque 96-well plate. Then, 10 µL of luciferase substrate was mixed with 500 µL of luciferase buffer and added to 4.5 mL of medium. Then, 50 µL of the mixture is pipetted into the white opaque 96-well plate and shaken for 3 min using a micromixer (TAITEC, E-36, Japan). The luminescence was measured using a multimode microplate reader (Berthold Technologies, Mithras LB 940, Germany). Cell viability was determined using 7.5% WST-8 and incubated for 4 h. The absorbance was then measured using a microplate reader at 450 nm. DMSO was used as a negative control, and Asteltoxin was included as a positive reference control [45].

Statistical analysis

Data are shown as mean ± standard deviation (SD) from three independent experiments (n = 3) performed in triplicate. Statistical analysis for the anti-cancer assays was performed using one-way ANOVA followed by Tukey's HSD post hoc test for multiple comparisons. Analyses were performed separately for each cell line. For PANC-1, comparisons were made among control and treatment groups. For HT29/CD63-Nluc cells,

comparisons were performed only among treatment groups. Statistical significance was defined as $p < 0.05$. Formal testing for normality was not performed before analysis. For antioxidant assays, the IC_{50} values for DPPH and ABTS, along with correlation analysis, were obtained using Microsoft Excel 2019. For anti-austerity analysis, the area under the relative viability curve (AUC), which is the sum of the measured responses (relative viability), was calculated with GraphPad Prism 10 (San Diego, CA, USA) to assess the selectivity of the compounds in targeting PANC-1 cells under glucose-starved conditions.

Results and discussion

Extraction yield

In this study, the flesh and peel were extracted using an ultrasonication-assisted extraction method using *n*-hexane, 80% EtOH, and water as solvents. As shown in **Table 1**, it can be observed that both the flesh and peel extracted using 80% EtOH as the extraction solvent exhibited the highest yield percentage compared to using *n*-hexane and water. Overall, the maximum extraction yield percentage obtained was 63.8%, which was achieved with the 80% EtOH extract of the flesh, and the water extract of the flesh displayed the minimum extraction yield of 2.7%. This suggests that 80% EtOH is a more efficient solvent for extracting bioactive compounds from *G. forbesii*. When compared to Muthia *et al.*, the fruit skin of *G. forbesii* extracted using 30% and 70% EtOH via the Soxhlet method produced a percentage yield of 21.99% and 17.22%, respectively [46]. In addition, similar findings can be found in a study reported by Núñez *et al*., [47] where *Tagetes erecta* flower extracts were extracted using an ultrasonically-assisted method with ethanol as the solvent. The author stated that the ultrasonicated-assisted method yielded twice as much as the Soxhlet method [47].

Table 1 The percentage yield of all the flesh and peel extracted using various solvents.

Sample extract	Solvent	Percentage yield (%)
Flesh	*n*-hexane	7.3
	80% EtOH	63.8
	Water	2.7
Peel	*n*-hexane	9.5
	80% EtOH	49.1
	Water	4.8

Qualitative phytochemical screening

To determine the phytochemicals present in the flesh and peel extracts of *G. forbesii*, the different extracts were tested for the presence of alkaloids, terpenoids, saponins, tannins, anthraquinone glycosides, and glycosides. **Table 2** shows the qualitative analysis of the phytochemical screenings, where the "+" symbol was used to denote the presence of these phytochemicals, whereas the "–" symbol indicated their absence. The phytochemical analysis revealed that no alkaloids were present in the flesh and peel extracts. However, terpenoids were detected in all of the flesh and peel. The presence of saponin can be found in the 80% EtOH extract of the flesh and peel. The tannins were also detected in the 80% EtOH extract of the flesh and peel. Moreover, it was observed that no anthraquinone glycoside was detected in all of the crude extracts.

Table 2 Results of the phytochemical constituents present in the flesh and peel of *G. forbesii*, where '+' indicates the presence of the compounds, whereas '−' states otherwise.

Extract	Solvent	Alkaloid	Terpenoid	Saponin	Tannin	Anthraquinone glycoside	Glycoside
Flesh	*n*-hexane	−	+	−	−	−	−
	80% EtOH	−	+	+	+	−	−
	Water	−	+	−	−	−	−
Peel	*n*-hexane	−	+	−	−	−	−
	80% EtOH	−	+	+	+	−	−
	Water	−	+	−	−	−	−

Total phenolic and flavonoid content

The phenolic and flavonoid compounds are known for their redox properties, contributing significantly to their antioxidant activity. This study evaluated the total phenolic content (TPC) and the total flavonoid content (TFC) of *n*-hexane, 80% EtOH, and water extracts from the flesh and peel of *G. forbesii*.

Using the equation of a standard calibration curve, $y = 0.0068x + 0.0408$ ($R^2 = 0.9999$) of gallic acid, the TPC results were expressed as mg of gallic acid equivalents (GAE) per g of dry extract (DE). The calculated TPC of all the extracts from the flesh and peel of *G. forbesii* can be found in **Table 3**. For the flesh, the 80% EtOH extract showed the highest phenolic content (16.51 ± 0.54 mg GAE/g), followed by *n*-hexane (3.86 ± 0.72 mg GAE/g) and water extract (3.02 ± 0.20 mg GAE/g). Additionally, the *n*-hexane extract of the peel displayed the highest phenolic content (27.31 ± 0.82 mg GAE/g), while the water extract had the lowest content (10.56 ± 0.51 mg GAE/g). Among all the extracts, the *n*-hexane extract of the peel exhibited the highest TPC compared to the flesh part. These findings suggest that phenolic compounds in the flesh of *G. forbesii* are primarily accumulated in the polar extracts. In contrast, phenolic compounds in the peel are mainly found in the non-polar extract.

Table 3 The TPC and TFC values of all extracts of the flesh and peel part of *G. forbesii*.

Parts of plant	Sample extract	TPC (mg GAE/g DE)	TFC (mg QE/g DE)
Flesh	*n*-hexane	3.9 ± 0.7	4.5 ± 0.9
	80% EtOH	16.5 ± 0.5	1.7 ± 0.9
	Water	3.0 ± 0.2	1.1 ± 0.9
Peel	*n*-hexane	27.3 ± 0.8	1.3 ± 0.9
	80% EtOH	25.8 ± 0.5	5.5 ± 0.5
	Water	10.6 ± 0.51	1.6 ± 1.2

The total flavonoid content (TFC) of all extracts was determined from the standard calibration curve, $y = 0.0082 + 0.0496$ ($R2 = 0.9999$) for quercetin and expressed as mg of quercetin equivalents (QE) per g of DE (dry extract weight). The calculated TFC of all the extracts of flesh and peel of *G. forbesii* can be shown in **Table 3**. Amongst all the extracts of the flesh part, the *n*-hexane extract exhibited the highest flavonoid content (4.45 ± 0.90 mg QE/g), followed by 80% EtOH (1.69 ± 0.90 mg QE/g) and water extract (1.07 ± 0.90 mg QE/g). For the peel part, the 80% EtOH extract had the highest flavonoid content (5.54 ± 0.50 mg QE/g), and the *n*-hexane extract had the lowest (1.25 ± 0.90 mg QE/g). Similar findings were reported by Ambarwati *et al*., [8] the flavonoid content of 70% ethanolic fruit peel extract was 1.96 ± 0.36 mg QE/g. Based on these results, the *n*-hexane extract of the peel showed the highest flavonoid content among the extracts. Hence, it can be indicated that the flavonoid compounds of flesh were accumulated

in the non-polar extract, whereas the peel was in the polar extract.

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH radical-scavenging assay is a method for assessing antioxidant activity. The assay is based on the transfer of a hydrogen atom or an electron from the scavenging molecule, which reduces the purple chromogen DPPH to form pale yellow hydrazine. The ability of the plant extracts to scavenge free radicals was assessed as % inhibition or % scavenging of DPPH. Furthermore, the free radical-scavenging activity will be calculated as the IC_{50}, where the IC_{50} is the concentration at which 50% of DPPH radicals are inhibited. From the graphs, IC_{50} values can be obtained using the linear equation derived from plots of the percentage of DPPH radical inhibition versus gallic acid concentration. **Figures 2(a)** and **2(b)** show linear plots of % inhibition versus concentration for the various flesh and peel extracts, respectively. It can be observed that all of the flesh and peel extracts are concentration-dependent, as the concentration increases, the percentage of inhibition also increases.

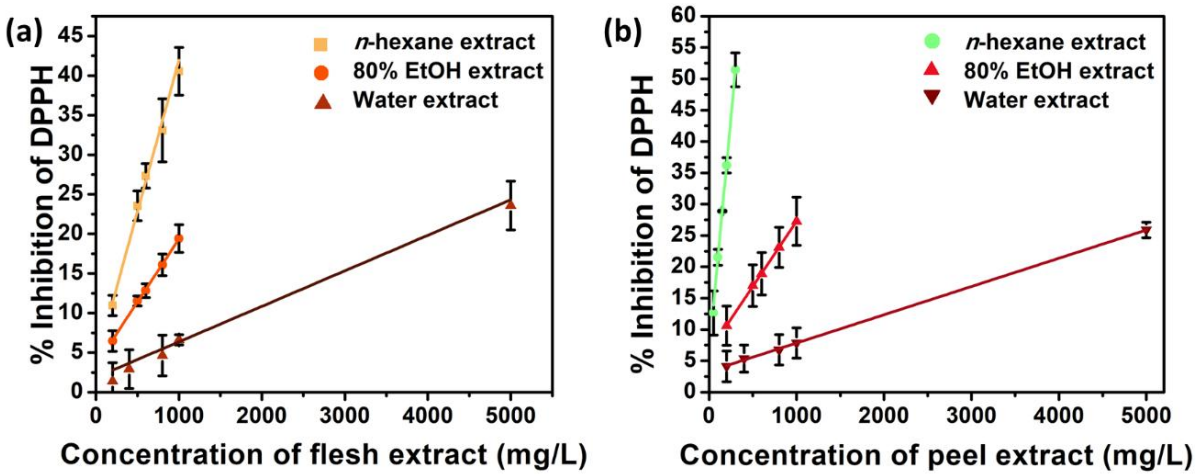


Figure 2 Plotted graphs of the percentage of DPPH radical inhibition (%) against concentrations of the (a) flesh and (b) peel extracts obtained using n-hexane, 80% EtOH, and water. Results are presented as the mean ± SD, and the error bars represent SD.

Table 4 displays the calculated percentage inhibition at a specific concentration and the IC_{50} for all flesh and peel extracts. Furthermore, it shows the % inhibition of the flesh part at a concentration of 1,000 mg/L. The *n*-hexane flesh extract showed the highest antioxidant activity of 40.5 ± 3.0% at a concentration of 1,000 mg/L, followed by 80% EtOH (19.4 ± 1.7%), and water extract (23.6 ± 3.1%). The % inhibition of peel extracts at a concentration of 200 ppm shows that the *n*-hexane extract had the highest % of inhibition (51.4 ± 2.7%), followed by the 80% EtOH extract, and the water extract. Moreover, among all extracts, the *n*-hexane extract from the peel showed the highest DPPH radical activity, with an IC_{50} of 289.4 mg/L. Our *n*-hexane peel extract showed higher antioxidant activity than that reported by Muthia *et al*., in which the 70% ethanolic rind extract of *G. forbesii* exhibited an IC_{50} of 534.60 ppm [46]. Furthermore, Ambarwati *et al.* also reported a lower DPPH antioxidant activity of the 70% ethanolic fruit skin of *G. forbesii* (IC_{50} value of 481.948 ppm) [8]. In comparison, the IC_{50} value of the n-hexane peel extract was lower, suggesting that our *n*-hexane extracts possessed higher antioxidant activity.

Table 4 Percentages of inhibition of DPPH at specific concentrations and the IC_{50} of all the flesh and peel extracts of *G. forbesii*.

Parts of plant	Sample extract	DPPH Inhibition (%)	IC_{50} (mg/L)
Flesh (1,000 mg/L)	*n*-hexane	40.5 ± 3.0	1,239.9
	80% EtOH	19.4 ± 1.7	2,899.9
	Water	23.6 ± 3.1	10,854.4
Peel (200 mg/L)	*n*-hexane	36.2 ± 1.2	289.4
	80% EtOH	10.6 ± 3.1	2,091.3
	Water	4.1 ± 2.5	10,373.7

Statistical analysis was also performed, the correlation analysis showed that the correlation coefficient (R) between TPC and IC_{50} of DPPH of the flesh and peel extract is R = 0.1781 and R = 0.9931, respectively. The high correlation observed for the peel extract indicates a possible association between the DPPH radical scavenging activity and the phenolic contents. Moreover, there was no apparent correlation between TFC and DPPH of the peel extracts, whereas the flesh extract showed a weak correlation with R = 0.5563. These findings may suggest that the phenolic compounds contribute to the DPPH radical scavenging activity of the extracts. In addition, the low correlation coefficient may be due to the non-polyphenolic compounds such as unsaturated fatty acids, carotenoids, and vitamins present in the extract that also contribute to the antioxidant activity, which affects the DPPH radical scavenging activity.

Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay is also used to examine the antioxidant capacity of extracts from the flesh and peel extracts of *G. forbesii*. The assay involves the reduction of Fe^{3+}/ferricyanide complex to Fe^{2+}/ferrous, producing a dark blue colour that can be monitored at a wavelength of 765 nm [48]. This reduction reaction indicates the presence of antioxidants in the sample. Moreover, the absorbance increases proportionately as the antioxidant capacity increases in reducing Fe^{3+} ions [49]. The results for all extracts were derived from the standard calibration curve, y = 0.0036 + 0.1266 (R^2 = 0.9998) for Trolox and expressed as mg of Trolox equivalents (TE) per g of DE (dry extract). The FRAP values of the flesh and peel extracts are presented in **Table 5**. The 80% EtOH extract of the flesh showed the highest FRAP value of 35.6 ± 3.8 mg TE/g, and the *n*-hexane extract showed the lowest FRAP value of 17.0 ± 1.5 mg TE/g. In contrast, among the peel extracts, *n*-hexane exhibited the highest FRAP value of 57.0 ± 0.9 mg TE/g, followed by the 80% EtOH extract (50.8 ± 0.6 mg TE/g) and the water extract (43.1 ± 0.5 mg TE/g). Overall, the peel extracts displayed higher FRAP values compared to the flesh extracts.

Table 5 FRAP value of the extracts using the flesh and peel of *G. forbesii*.

Sample extract	FRAP (mg TE/g DE)		
	***n*-hexane**	**80% EtOH**	**Water**
Flesh	17.0 ± 1.5	35.6 ± 3.8	26.7 ± 0.8
Peel	57.0 ± 0.9	50.8 ± 0.6	43.1 ± 0.5

The correlation coefficients between TPC and FRAP for the flesh and peel extracts were R = 0.6775 and R = 0.8632, respectively. A high correlation between TPC and FRAP was observed for the peel. Between TFC and FRAP, the peel extract showed no significant correlation, whilst the flesh had a moderate correlation (R = 0.6108). The TPC-FRAP relationship exhibited higher correlation coefficient values than the

TFC-FRAP relationship, suggesting that the phenolic compounds may contribute substantially to the reducing antioxidant activity more than the flavonoid compounds in the extracts. Furthermore, differences in the composition, concentration, and specific structures of phenolic and flavonoid compounds in the extracts might contribute to the variation in correlation strength [38,50].

2,2′-azinobis 3-ethylbenzothiazoline 6-sulfonate (ABTS) assay

The ABTS chemical assay was also conducted to determine the antioxidant activities of the *G. forbesii* plant extracts. The basis of the ABTS assay involves the decolourisation of pale green-blue, indicating the presence of an antioxidant in the samples. **Figures 3(a)** and **3(b)** showed a graph of % inhibition of ABTS against the concentration of the flesh and peel extracts, respectively. It can be seen that the flesh and peel extracts are concentration-dependent. In **Figure 4(a)**, the *n*-hexane extract of the flesh showed the highest % inhibition of 11.7 ± 3.0%, followed by 80% EtOH (5.3 ± 0.9%), and water extract (1.9 ± 0.6%) at the concentration of 200 mg/L. For the peel extract, the *n*-hexane exhibited the highest antioxidant activity (39.2 ± 1.1%) with the water extract having the lowest activity (6.8 ± 2.3%) at a concentration of 100 mg/L, as shown in **Figure 4(b)**. Among all extracts, the *n*-hexane extract from the peel showed the highest antioxidant activity with an IC_{50} value of 125.0 mg/L. **Table 6** displays the calculated ABTS percentage of inhibition at various concentrations and IC_{50} values for all the extracts.

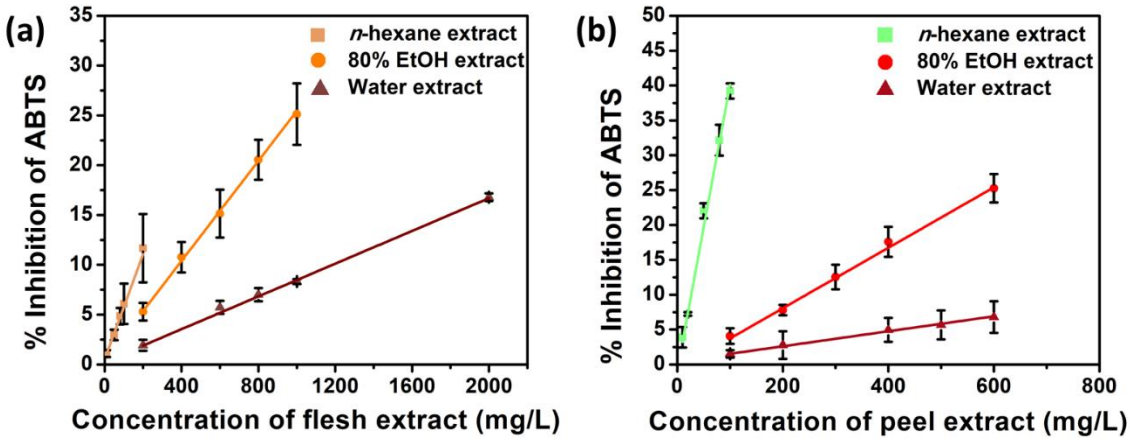


Figure 3 Plotted graphs of the percentage of ABTS inhibition (% inhibition) of the (a) flesh and (b) peel extracted using *n*-hexane, 80% EtOH, and water at different concentrations. Results are expressed as mean ± SD, and the error bars represent SD.

Table 6 ABTS percentage inhibition at a specific concentration and IC_{50} value of all extracts.

Parts of plant	Sample extract	ABTS Inhibition (%)	IC_{50} (mg/L)
Flesh (200 mg/L)	*n*-hexane	11.7 ± 3.0	867.0
	80% EtOH	5.3 ± 0.9	1,981.7
	Water	1.9 ± 0.6	5,991.0
Peel (100 mg/L)	*n*-hexane	39.2 ± 1.1	125.0
	80% EtOH	4.1 ± 1.1	1,163.4
	Water	1.5 ± 0.5	45,169.0

The regression analysis revealed that the correlation coefficients between TPC and ABTS for the flesh and peel were 0.1312 and 0.9958, respectively. There was a high correlation between TPC and the ABTS of the peel extract, suggesting a possible association between the phenolic compounds and the antioxidant activity in the peel extract. In addition, the correlation coefficient between TFC and ABTS of all

the extracts was also obtained. The TFC and ABTS of the flesh extract (R = 0.6202) showed threefold higher correlation compared to the peel (R = 0.1736). Hence, the observed correlation suggests that the flavonoid compounds may contribute to the ABTS scavenging activity of the flesh extract.

Evaluation of anti-austerity activity against PANC-1 cells

PANC-1 cells are a suitable model to investigate an anti-austerity approach due to their innate tolerance to glucose starvation, which allows the cancer cells to thrive in hypervascular tumor microenvironments deficient in oxygen and nutrients [3,20,21,42]. This phenomenon is known as "austerity". Therefore, the development of anti-austerity agents that specifically target cancer cells when they are deprived of nutrients is a promising strategy in treating pancreatic cancer cells in their microenvironment [3,20]. Moreover, the anti-austerity agents are less harmful to nutrient-rich cancer cells, hence, they should have fewer adverse effects than conventional anticancer drug treatments [3,51].

In this study, all flesh and peel extracts of *G. forbesii* were tested for cytotoxicity against PANC-1 cells under normal and glucose-free conditions. As shown in **Table 7**, the *n*-hexane extract of the peel exhibited strong cytotoxicity toward PANC-1 cells under glucose-starved conditions, reducing cell viability to 27% at 10 µg/mL, whereas it had minimal effect under normal conditions, where cell viability remained ≥80%. However, the 80% EtOH and water extracts of the peel showed no cytotoxic activity under glucose-starved conditions. To evaluate the selectivity of the extracts, the difference in cell viability between the 2 media was compared and used as a metric, with a ≥50% difference considered high selectivity. No selectivity was observed with the 80% EtOH and water extracts of the flesh and peel. The *n*-hexane extract of the flesh demonstrated moderate selectivity, with viability differences of 41.24%. In contrast, the *n*-hexane peel extract showed the highest selectivity, with a viability difference of 60.35%. These results suggest that the *n*-hexane peel extract may possess anti-austerity properties and could serve as a potential anti-austerity agent.

Table 7 The percentage of cell viability of the PANC-1 cells after being treated with the sample extract in normal glucose and glucose-starved conditions.

Sample extract	Solvent	Anti-austerity (% Cell viability ± SD) at 10 µg/mL		Selectivity
		Glucose (-ve)	Glucose (+ve)	
Flesh	*n*-hexane	34.6 ± 10.1	75.9 ± 7.8	Selective
	80% EtOH	100.9 ± 7.5	100.2 ± 9.3	Not selective
	Water	125.2 ± 2.7	106.2 ± 6.5	Not selective
Peel	*n*-hexane	27.0 ± 4.3	87.4 ± 4.2	Selective
	80% EtOH	101.6 ± 10.4	84.1 ± 3.7	Not selective
	Water	96.9 ± 8.8	87.5 ± 3.0	Not selective

To further confirm the selectivity of the extracts, a dose-response parameter, the area under the relative viability curve (AUC), defined as the sum of the measured responses (relative viability) across all tested concentrations of all the extracts, was calculated using GraphPad Prism 10 [52]. Lower AUC values indicate greater cytotoxicity, and under glucose-deprived conditions in PANC-1 cells, this corresponds to higher anti-austerity activity [52,53]. Hence, the compound was categorised as selective. As shown in **Table 8**, the AUC values were normalised to the largest AUC to enable comparisons between samples under normal-glucose and no-glucose conditions. A normalised AUC value of 1.0 indicates minimal activity, whereas lower values indicate higher cytotoxic activity [39]. Subsequently, the *n*-hexane extract of the flesh and peel of *G. forbesii* showed significantly lower AUC values in the nutrient-starved conditions of 370.3 and 305,

respectively, compared to their normal glucose conditions. After normalisation, the AUCs of the *n*-hexane extracts of flesh and peel were 0.49 and 0.37, respectively, suggesting enhanced cytotoxicity under no-glucose conditions and potential anti-austerity activity. In contrast, the AUC values of the 80% EtOH and water extracts of the flesh and peel showed lower cytotoxic effects under no-glucose conditions, even after normalisation, reflecting non-selective compounds. Among all extracts, the *n*-hexane extract of the peel demonstrated the lowest normalised AUC, indicating greater selectivity for PANC-1 cells under glucose starvation. Glucose starvation induces metabolic stress pathways that normally promote cell survival [20,21]. This selective cytotoxicity under glucose-starved conditions may indicate that the active constituents of the extracts interfere with cellular survival pathways activated during metabolic stress, thereby reducing the ability of cancer cells to tolerate nutrient deprivation [20,21]. However, the underlying mechanism remains to be elucidated. The higher activity observed in the peel *n*-hexane extract compared to 80% EtOH and water may be due to the differences in phytochemical composition. Solvent polarity may significantly influence the bioactive compounds extracted, with non-polar solvents generally enriching lipophilic constituents such as terpenoids and other hydrophobic metabolites, whereas polar solvents recover hydrophilic compounds such as phenolics and flavonoids [54]. The differences in biological activities of these phytochemical classes may therefore contribute to the higher activity observed in the *n*-hexane extract [54]. Further phytochemical characterisation is required to identify the compounds responsible for the effects. Overall, *G. forbesii* peel *n*-hexane extract shows preliminary evidence of anti-austerity activity and warrants further investigation into its potential application under glucose-starved conditions.

Table 8 Compilation of the total AUC raw data and the normalised AUC data in two different media.

Sample extract	Solvent	Total AUC (raw)		Normalized AUC		Selectivity
		Glucose (-ve)	Glucose (+ve)	Glucose (-ve)	Glucose (+ve)	
Flesh	*n*-hexane	370.3	753.1	**0.49***	1.00	Selective
	80% EtOH	1,441.0	935.9	1.00	0.65	Not selective
	Water	1,129.0	996.1	1.00	0.88	Not selective
Peel	*n*-hexane	305.0	833	**0.37***	1.00	Selective
	80% EtOH	9,143.0	8582	1.00	0.94	Not selective
	Water	885.9	827.7	1.00	0.93	Not selective

Note: *indicates a statistically significant difference compared with the control group ($p < 0.05$).

Evaluation of cytotoxicity and inhibition of EV production in HT-29/CD63-Nluc cells

Extracellular vesicles (EV), including exosomes, are small membranous structures that facilitate intercellular communication by transporting intracellular components such as proteins and ribonucleic acid (RNA) [55,56]. These vesicles are produced by various cell types and are found in bodily fluids, including blood, urine, and saliva. They can be identified by their distinct mechanisms of biogenesis and secretion [55-57]. In cancer research, tumor-derived exosomes carry oncogenic proteins and nucleic acids that modulate the tumor microenvironment, promoting conditions favorable for tumor growth [22,58,59]. Compared to normal cells, cancer cells release more EV [23,60]. These tumor-derived EV promote angiogenesis, metastasis, and cell proliferation in target cells, thereby accelerating cancer progression [22-24]. Therefore, inhibiting EV production may suppress tumor growth and metastasis. In this study, cytotoxicity and luciferase assays were performed on HT29/CD63-Nluc cells to measure cytotoxicity and EV production, respectively. The HT-29/CD63-Nluc cells are stable transformants that secrete EV, including CD63 fused to luciferase [61]. As a result, the amount of EV correlates with luciferase activity [61].

Figures 4(a) and **4(b)** display the luminescence intensity and cell viability of HT29/CD63-Nluc cells treated with the flesh extracts. As the *n*-hexane flesh extract concentration increases from 10 to 100 µg/mL, luminescence intensity rises, but cell viability drops to nearly zero at 100 µg/mL, reflecting high toxicity and non-selectivity of the compound. The increase in luminescence intensity despite cell death may result from loss of membrane integrity, as dying cells release intracellular CD63–luciferase fusion protein, which paradoxically elevates luminescence activity [25,62-64]. In contrast, the 80% EtOH and water flesh extracts show minimal cytotoxicity, but their luminescence activity does not show concentration-dependent inhibition of EV secretion. Therefore, it can be inferred that all of the flesh extracts are non-selective in inhibiting EV secretion. In comparison, Asteltoxin has been reported to strongly suppress EV secretion at 10 µg/mL without inducing cell death, acting through AMPK/mTORC1-mediated lysosomal activation [45]. This concentration thus serves as a positive control for selective EV inhibition. Additionally, the peel *n*-hexane extracts show the strongest EV suppression at higher concentrations than the 80% EtOH and water extracts. However, this effect is largely attributable to cytotoxicity, which reduces cell viability, indicating non-selectivity of the compound. In contrast to Asteltoxin at 10 µg/mL which suppresses EV secretion without cytotoxicity [45], the EV inhibition of the *n*-hexane peel extract is largely attributable to cell death rather than an EV-regulatory mechanism. In this case, further testing at lower concentrations is necessary to determine whether the peel *n*-hexane extract can inhibit EV secretion independently of toxicity. Subsequently, the 80% EtOH extract of the peel maintained high cell viability and demonstrated moderate EV inhibition. The peel water extract shows only a minor effect on luminescence levels. The 80% EtOH extract of the peel demonstrates promising potential as a selective EV secretion inhibitor without inducing cell death, although the magnitude of EV suppression was lower than that reported for Asteltoxin at 10 µg/mL [45]. This activity suggests that polar bioactive compounds may contribute to EV modulation. The reduction in EV secretion may be linked to modulation of stress-related pathways that regulate vesicle formation and release, though the specific mechanism behind this effect remains unclear.

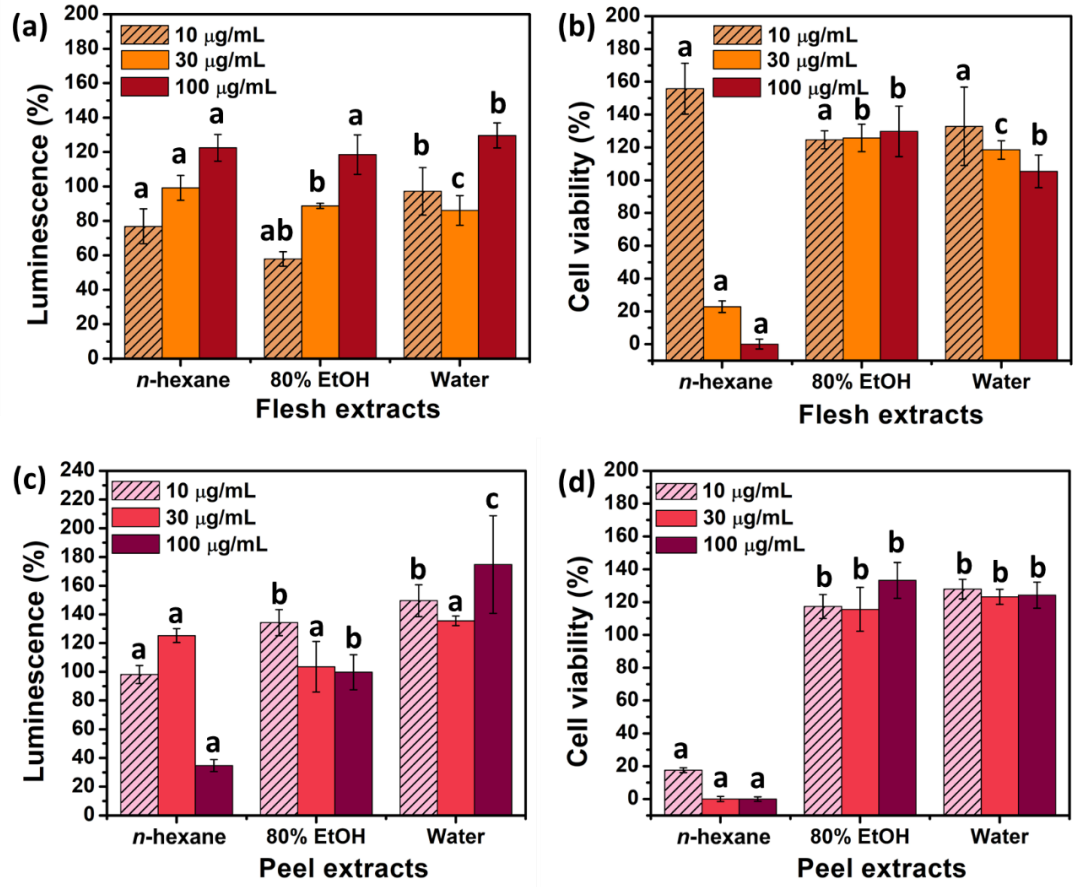


Figure 4 The HT-29/CD63-Nluc cells were treated with all of the sample extracts using concentrations of 10 - 100 μg/mL, where (a) and (c) show the effect of EV production in HT29/CD63-Nluc cells and (b) and (d) show the cell viability when treated with *n*-hexane, 80% EtOH, and water extracts of flesh and peel, respectively. Results are expressed as mean ± SD, and the error bars represent SD. Statistical comparisons were made between the extracts (*n*-hexane, 80% EtOH, and water) within each concentration group. Different lowercase letters indicate statistically significant differences between treatments at $p < 0.05$, whereas bars sharing the same letter in the same group concentration are not significantly different.

Despite these promising results, this study is limited by its in *vitro* design, which may not fully reflect the complexity of tumor progression *in vivo*. Therefore, further *in vivo* studies are required to validate the anti-austerity and EV inhibitory activities observed and to assess their therapeutic potential.

Conclusions

In this study, the flesh and peel of *G. forbesii* were successfully sequentially extracted via an ultrasonic-assisted maceration method using *n*-hexane, 80% EtOH, and water as solvents. Overall, 80% EtOH appears to be a more efficient solvent for extracting bioactive compounds from *G. forbesii.* Both the flesh and peel extracts contain terpenoids, saponins, and tannins. Among all extracts, the *n*-hexane peel extract showed moderate antioxidant activity in DPPH and ABTS assays, with IC_{50} values of 289.4 and 125.0 mg/L, respectively, whereas the FRAP value was 57.0 ± 0.9 mg TE/g DE. According to statistical analysis, the phenolic compounds in the peel extract are the primary contributors to the antioxidant activities. Among all extracts, the *n*-hexane peel extract had the highest antioxidant activity in DPPH and ABTS assays, with IC_{50} values of 289.4 and 125.0 mg/L, respectively, whereas the FRAP value was 57.0 ± 0.9 mg TE/g DE. According to statistical analysis, the phenolic compounds in the peel extract are the primary contributors to the antioxidant activities. Moreover, the *n*-hexane extract of the peel exhibited the highest selective cytotoxicity against PANC-1 cells under glucose-starved conditions, yielding the smallest AUC

value (0.37). In addition, the 80% EtOH extract of the peel shows selective inhibition of EV secretion without compromising cell viability, suggesting possible modulation of EV-mediated cancer cell communication. Overall, the *n*-hexane and 80% EtOH peel extracts exhibit selective activities relevant to cancer cell stress responses under *in vitro* conditions. These results suggest that *G. forbesii* extracts may represent promising sources of bioactive compounds with antioxidant and anticancer activities. However, further *in vivo*, mechanistic, and toxicity studies are required to validate their biological significance and potential therapeutic relevance.

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Declaration of Generative AI In Scientific Writing

The authors acknowledge that generative AI tools such as Grammarly and OpenAI's ChatGPT were used solely for language refinement and grammar editing during the preparation of this manuscript. No part of the content was generated by AI, nor was any data interpreted by it. The authors accept full responsibility for the content and conclusions of this work.

CRediT Author Statement

Asyiqin Zulkiflee: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Norhayati Ahmad**: Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Peng Du**: Writing – review & editing, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Masayoshi Arai**: Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Hartini Mohd Yasin**: Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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