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Investigation of Antimicrobial, Antioxidant, and Cytotoxic Activities of *Boesenbergia rotunda* rhizome extract

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Abstract

In recent years, antimicrobial resistance (AMR) has become a global threat to public health. In Hawai'i, there is an increasing rate of staph infections of methicillin-resistant *Staphylococcus aureus* (MRSA), and, hence, a need for new agents to combat the increase of AMR bacteria. This study evaluates the antimicrobial, antioxidant, and cytotoxic activities of Hawaiian-grown *Boesenbergia rotunda*. Test bacteria included clinical isolates of Gram-positive MRSA and methicillin-susceptible *S. aureus* (MSSA) as well as Gram-negative *Serratia marcescens* and *Escherichia coli*. Five compounds–cardamonin, pinostrobin, pinocembrin, pinostrobin chalcone, and isopanduratin A–were isolated from the ethyl acetate extract of *B. rotunda* rhizome, and their structures were identified by NMR spectroscopy. These samples exhibited antimicrobial activity against MRSA and MSSA strains, with minimum inhibitory concentration (MIC) values between 128 and 2 µg/mL, with isopanduratin A giving MIC values as low as 2 µg/mL. The antioxidant potential of samples was examined using ferric-reducing antioxidant power (FRAP) assay. At 1 mg/mL of tested samples, FRAP values ranged between 8.74 to 17.76 µM/µg, with pinostrobin chalcone exhibiting the highest FRAP value (17.76 ± 0.65 µM/µg). Moreover, cytotoxicity was measured via sulforhodamine B (SRB) assay. Cardamonin (IC_{50} of 19.43 ± 0.33 µM) and isopanduratin A (IC_{50} of 26.84 ± 1.06 µM) exhibited effectiveness against the lung cancer cell line A549. Compounds from *B. rotunda* showed potent antimicrobial effect against MRSA and MSSA strains as well as antioxidant and cytotoxic activities, and may have the potential for further evaluation and development for pharmaceutical applications.

Keywords**:** *antimicrobial; antioxidant; cytotoxicity; natural products; Boesenbergia rotunda.*

1. Introduction

Staphylococcus aureus, a Gram-positive bacterium, is a leading cause of skin infections worldwide and globally (Boucher, & Corey, 2008; Chen, & Huang, 2014). Methicillin-susceptible *S. aureus* (MSSA) infections are treated with antibiotics such as cefazolin, oxacillin, and, in severe cases, vancomycin (Rayner, & Munckhof, 2005). However, the over-prescription and overuse of these antimicrobial drugs have led to the rapid emergence of methicillin-resistant *S. aureus* (MRSA) (Fukunaga et al., 2016). The danger in these strains primarily lies in their resistance to antibiotics, and consequently, the risk of mortality associated with hospital-acquired staph infections has increased drastically, especially during the COVID-19 pandemic (Boucher, & Corey, 2008; Adalbert et al., 2021). With high proportions of MRSA endemic to

healthcare facilities in both the United States and countries throughout Asia, both known strains of MRSA and the continuous development of new resistance strains are global health issues, necessitating the discovery of new antimicrobial agents (Boucher, & Corey, 2008; Chen, & Huang, 2014).

B. rotunda (Family: Zingiberaceae) is a species of ginger unique to a region encompassing Southeast Asia, South Asia, and Southern China (Ongwisespaiboon, & Jiraungkoorskul, 2017). It is commonly known as the Chinese keys, Chinese ginger, or fingerroot due to its characteristic shape of several fingerlike rhizomes extending from a focal point. *B. rotunda* ranges between 15 to 40 centimeters in length and 1.0 to 2.0 centimeters in diameter (Eng-Chong et al., 2012). *B. rotunda* is commonly used for relieving coughs and as a flavor enhancer in traditional cuisine (Tang et al., 2007). However, it has recently garnered attention for its biological properties due to its abundance of phytochemicals, including flavonoids such as cardamonin and panduratin (Yusuf et al., 2013); essential oils such as camphor and limonene (Baharudin et al., 2015); and polyphenols such as caffeic acid, and hesperidin, among others (Jing et al., 2010).

There are several studies of medicinal plants with good antimicrobial properties (Mahesh, & Satish, 2008). However, there has yet to be a study quantifying the antimicrobial activity of Hawaiian-grown *B. rotunda* rhizomes on strains of MRSA isolated from Hawai'i, which have become more prevalent today (Gerken et al., 2021). Similarly, while previous studies have documented the antioxidant capacity of the methanolic extract of *B. rotunda* rhizome, only a few reports have investigated the antioxidant properties of compounds isolated from the *B. rotunda* rhizome (Jing et al., 2010).

Therefore, this study aims to isolate and characterize natural products from Hawaiian-grown *B. rotunda* and evaluate their antimicrobial, antioxidant, and cytotoxic activities.

2. Objectives

The main research objectives of this work were to extract, isolate, purify, and identify the chemical constituents from the extract of *B. rotunda* rhizome. All compounds were evaluated for their antimicrobial, antioxidant, and cytotoxic activities on A549 human lung cancer cell line.

3. Materials and methods

3.1 General Experimental Procedures

Ultraviolet spectra were measured using a Shimadzu PharmaSpec-1800 UV-visible spectrophotometer (Shimadzu Scientific Instruments, MD, USA). 1D- and 2D-nuclear magnetic resonance (NMR) spectra were measured using a Bruker ADVANCE DRX-400 MHz spectrometer (Bruker, Billerica, MA, USA). Spectra obtained was processed using TopSpin 3.2 software. Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} (0.25 mm, Merck, Darmstadt, Germany). Silica gel (230-400 mesh, 480-800 mesh, Sorbent Technologies, Atlanta, GA, USA) and Sephadex LH-20 (GE Healthcare, Piscataway, NJ, USA) were used for column chromatography.

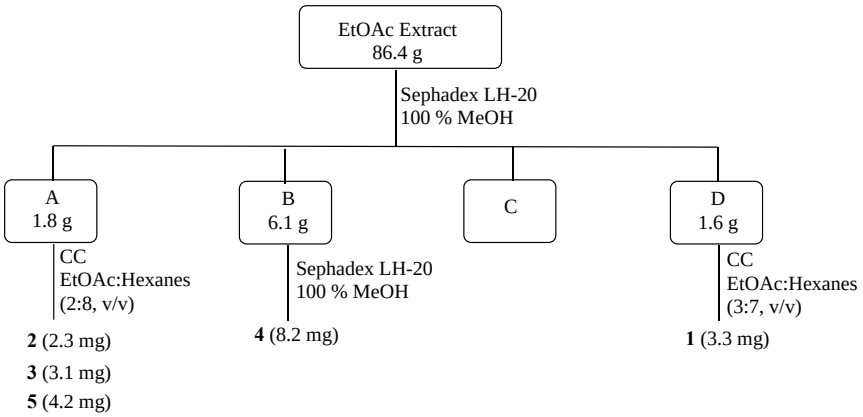
3.2 Plant Materials

B. rotunda was grown in a greenhouse at the Daniel K. Inouye U.S. Pacific Basin Agricultural Research Center, Hilo, Hawai'i, USA, in August 2021 and was provided by Dr. Marisa M. Wall. Voucher specimens (No. BFR001) were stored at the Natural Product Chemistry Laboratory, Department of Pharmaceutical Sciences, Daniel K. Inouye College of Pharmacy, University of Hawai'i at Hilo.

3.3 Extraction and Isolation

Freshly harvested *B. rotunda* rhizome was cleaned with fine-bristled brushes and rinsed in tap water to remove sediments from the plant. *B. rotunda* rhizomes were first air-dried and ground into a fine powder (4096.5 g) using a stainless-steel kitchen blender. The powder was extracted with ethyl acetate thrice (950 mL each time) at room temperature for 72 hours. The filtered ethyl acetate (EtOAc) extract was evaporated under reduced pressure to give a crude weight of about 86.4 g.

This crude extract was subjected to Sephadex LH-20 column chromatography (CC) (100% methanol) to afford main fractions (A-D). Fraction A (1.8 g) was separated by silica-gel column chromatography eluting with EtOAc-hexanes (2:8, v/v) to furnish pinostrobin (**2**) (2.3 mg), pinocembrin (**3**) (3.1 mg), and pinostrobin chalcone (**5**) (4.2 mg). Fraction B (6.1 g) was further purified on a Sephadex LH-20 CC (100% methanol) to yield cardamonin (**4**) (8.2 mg). Isopanduratin A (**1**) (3.3 mg) was obtained from fraction D (1.6 g) by silica gel column chromatography eluting with EtOAc-hexanes (3:7, v/v) (Scheme 1).



Scheme 1 Isolation of *B. rotunda* rhizome Extract

Isopanduratin A (**1**): yellow solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 14.04 (1H, s, 6-OH), 7.14–7.29 (5H, m, H-2‴–H-6‴), 6.01(1H, d, J = 2.4 Hz, H-3), 5.99 (1H, d, J = 2.4 Hz, H-5), 5.49 (1H, brt, H-4′), 4.92 (1H, t, J = 7.4 Hz, H-2″), 4.57 (1H, d, J = 11.4, 4.6 Hz, H-1′), 3.97 (3H, s, 2-OCH_3), 3.48 (1H, m, H-6′), 2.55 (1H, m, H-2′), 2.47 (1H, m, H-5′), 2.31 (2H, m, H-1″), 2.08 (1H, m, H-5′), 1.85 (3H, s, 3′-CH_3), 1.56 (6H, s, 4″-CH_3 and 5″-CH_3).

Pinostrobin (**2**): yellow solid; ^{1}H NMR (acetone-d_6, 400 MHz) δ 12.14 (1H, s, 5-OH), 7.39–7.59 (5H, m, H-2′–H-6′), 6.11(1H, d, J = 2.1 Hz, H-6), 6.08 (1H, d, J = 2.1 Hz, H-8), 5.60 (1H, dd, J = 12.7, 3.1 Hz, H-2), 3.87 (3H, s, 7-OCH_3), 3.20 (1H, dd, J = 17.4, 12.7 Hz, H-3b), 2.84 (1H, dd, J = 17.4, 3.1 Hz, H-3a).

Pinocembrin (**3**): yellow solid; ^{1}H NMR (acetone-d_6, 400 MHz) δ 12.19 (1H, s, 5-OH), 7.38–7.57 (5H, m, H-2′–H-6′), 6.03 (1H, d, J = 2.1 Hz, H-6), 6.01 (1H, d, J = 2.1 Hz, H-8), 5.55 (1H, dd, J = 12.7, 3.1 Hz, H-2), 3.16 (1H, dd, J = 17.4, 12.7 Hz, H-3b), 2.83 (1H, dd, J = 17.4, 3.1 Hz, H-3a).

Cardamonin (**4**): yellow solid; ^{1}H NMR (acetone-d_6, 400 MHz) δ 14.18 (1H, s, 2′-OH), 8.01 (1H, d, J = 15.7 Hz, H-3), 7.77 (1H, d, J = 15.7 Hz, H-2), 7.43–7.74 (5H, m, H-5–H-9), 6.10 (1H, d, J = 2.1 Hz, H-5′), 6.03 (1H, d, J = 2.1 Hz, H-3′), 3.99 (3H, s, 6′-OCH_3).

Pinostrobin chalcone (**5**): yellow solid; ^{1}H NMR (acetone-d_6, 400 MHz) δ 12.17 (1H, brs, 2′-OH), 8.25 (1H, dd, J = 15.6 Hz, H-3), 7.79 (1H, dd, J = 15.6 Hz, H-2), 7.43–7.72 (5H, m, H-5–H-9), 6.06 (2H, s, H-3′ and H-5′), 3.83 (3H, s, 4′-OCH_3).

3.4 Bacterial cultures

Methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-susceptible *S. aureus* (MSSA), and Gram-negative clinical isolates of bacteria (*Escherichia coli* (9637) and *Serratia marcescens* ATCC 13880) were used for this study. MRSA USA-300 LAC, MRSA *S. aureus* N315, *S. aureus* Newman, *S. aureus* 8325-4, *E. coli*, and *S. marcescens* were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). MSSA HBP10, MSSA LUU7, MSSA R113, *S. aureus* ONE9 and MSSA ONE6 were isolated from Hawai'i (Gerken et al., 2021). Mueller Hinton (MH) Broth and Mueller Hinton Agar were used for bacterial inoculation (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). *S. aureus* N315 was an MRSA pathogen strain causing hospital-acquired infections in 1982. This strain is easy to acquire antibiotic resistance, such as vancomycin. MSSA strains isolated from the Island of Hawaii include: Honolii Beach Park (HBP10), Wailuku River (LUU7): *bla*Z [β-lactam resistance] gene, Richardson's Beach Park (R113), and Onekahakaha (ONE6): contained antimicrobial resistance gene: *bla*Z (Gerken et al., 2021).

3.5 Disk Diffusion Method

The antimicrobial activity of *B. rotunda* rhizome extracts and samples was first evaluated by disk diffusion method against Gram-positive and Gram-negative bacteria (Bauer et al., 1966; Hudzicki, 2009). Overnight growth of each bacterium adjusted to the turbidity of 0.5 McFarland standard, was spread evenly onto the Mueller-Hinton agar plate in sterile condition. The concentration of each sample (1 mg/125 µL) was

prepared and dissolved in dimethyl sulfoxide (DMSO). Subsequently, 20 µL of the solution (160 µg of sample) was pipetted onto paper disks (diameter 7 mm) and transferred aseptically onto the agar plate. A solvent DMSO disk was a negative control; ampicillin disk (10 µg) was a positive control. The plates inoculated with Gram-positive and Gram-negative bacteria were incubated at 37 °C for 24 h. The microbial growth was determined by measuring the zone of inhibitions of each sample measured in millimeters.

3.6 Determination of minimum inhibitory concentrations (MIC)

The minimum inhibitory concentration (MIC) is the lowest concentration of a sample that inhibits bacterial growth. The MICs were determined by a 2-fold serial dilution method using Mueller Hinton broth (MHB) (Reller et al., 2009; Elshikh et al., 2016). Briefly, serial two-fold dilutions of samples in DMSO and then mixed with MHB in a 96-well microplate, and followed by the addition of 50 µL of bacteria to each well (final concentration of 1×10^4 colony forming units/well). The plates were incubated period of 18−24 hours at 35−37 °C followed by the addition of 20 µL of 0.018% resazurin. The MIC was determined after adding resazurin for 1-2 h. The experiments were performed in triplicate, and the standard antibiotics were vancomycin and gentamycin.

3.7 Ferric-Reducing Antioxidant power (FRAP) Assay

The FRAP assay determined the antioxidant activity of *B. rotunda* rhizome extracts and compounds using a modified version of the Pandey and Rizvi protocol. This assay measures a sample's ability to reduce ferric tripyridyltriazine [Fe(III)-TPTZ] to ferrous tripyridyltriazine [Fe(II)-TPTZ] and measuring the absorbance at 593 nm using a BioTek ELx800 Microplate Reader (BioTek Instruments Inc. Winooski, Vermont, USA). All FRAP absorbance values were transferred from the Microplate Reader through the Gen 5 computer software to Microsoft Excel for FRAP value calculations. 1.0 M ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) was serially diluted to 1000, 800, 600, 400, 200, 100, and 50 µM was made to plot a standard curve. The FRAP reagent was prepared (300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tris(2-pyridyl)-*S*-triazine) solution, and 20 mM ferric (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) in a 10:1:1 ratio, respectively) and heated to 37 ºC for ten minutes before use.

A blank reading was taken at 593 nm with 150 µL FRAP reagent added to each well of a 96-well microtiter plate. Next, 20 µL of each sample was added to wells in triplicate for readings at 593 nm. Samples were prepared at 1 mg/mL, 0.5 mg/mL, and 0.25 mg/mL, and ascorbic acid at 1 mg/mL served as a positive control. Reducing capacity was expressed by FRAP values in a µM $FeSO_4$/µg sample unit.

3.8 Cell Lines and Sulforhodamine B (SRB) Cytotoxicity Assay

A549 (CCL-185™) cells were purchased from the ATCC. These cells were cultured at 37 °C in a humidified incubator with 5% CO_2. High glucose DMEM supplemented with heat-inactivated fetal bovine serum (FBS, 10%) was used as the growth medium, with antibiotics penicillin (1000 units/mL) and streptomycin (1000 µg/mL).

B. rotunda extract and compounds were evaluated for cytotoxicity activity using an *in vitro* SRB assay against A549 lung cancer cells (Islam et al., 2023). A549 cells (6000 cells/well) were seeded in a 96-well plate for 24 hours followed by incubation for 72 hours at 37 ºC in the presence of increasing sample concentrations (1.25–50 µg/mL) or 0.5% DMSO (control). Cisplatin, at a concentration of 0.5 – 20 µM, was used as positive control. After the incubation, cells were fixed with 50 µL of 10% trichloroacetic acid for 60 minutes at 4 °C. Next, the plate was washed repeatedly with water, dried, and stained with 100 µL of 0.4% SRB in 1% acetic acid solution for 30 min. Then, the plate was washed repeatedly with 1% acetic acid to remove the excess dye and allowed to dry at room temperature. The protein-bound dye was dissolved in 200 µL of 10 mM Tris buffer (pH 10.0) and optical density was recorded at 515 nm. The experiments were performed in triplicate. The % mortality was calculated with the following equation:

$$\% \text{ Mortality} = \frac{(ODc - ODo) - (ODs - ODo)}{(ODc - ODo)} \times 100$$

OD_0 = Optical density of cells before adding sample/standard (0 day)

OD_C = Optical density of cells in control well at 72 hours

OD_S = Optical density of cells after 72 hours of incubation with sample/standard

The median inhibitory concentration (IC_{50}), at which 50% cellular mortality was observed, was calculated by plotting % mortality against sample concentration and non-linear curve fit analysis.

3.9 Statistical Analysis

All data were presented as mean ± standard error of the mean (SEM) and were obtained from three separate experiments. Statistical analyses were tested by Student's t-test or ANOVA followed by Tukey's multiple comparisons test; *p<0.05, **p<0.01 and ***p<0.001 were set statistically significant. IC_{50} values of A549 cells were calculated by non-linear curve fit analysis using Prism software (GraphPad 10.0.2, San Diego, USA) with R^2 >0.9 and P >0.5 (runs test) as parameters of goodness-of-fit.

4. Results and Discussion

4.1 Isolated compounds from *B. rotunda* rhizomes

The primary screen of antibacterial activities indicated that *B. rotunda* rhizomes EtOAc crude extract, fractions A, B and D were active. Therefore, repeated column chromatography of the EtOAc crude extract and fractions gave a total of five compounds including one cyclohexenyl chalcone; isopanduratin A (**1**) (Hwang et al., 2004), two flavanones; pinostrobin (**2**) (Smolarz et al., 2006), pinocembrin (**3**) (Maciejewicz, 2001), and two chalcones; cardamonin (**4**) (He et al., 2005), pinostrobin chalcone (**5**) (Malek et al., 2011) as shown in Figure 1. Structure identification of the isolated compounds was elucidated based on 1D- and 2D NMR spectra spectroscopy.

Compound **1** was obtained as yellow solids. The ^{1}H NMR spectrum displayed the presence of a set of hydrogen-bonded hydroxy group at δ_H 14.04 (1H, s, 6-OH) and a monosubstituted benzene ring at δ_H 7.14–7.29 (5H, m, H-2‴–H-6‴). Two coupled doublet resonances at δ_H 6.01 (1H, d, *J* = 2.4 Hz) and 5.99 (1H, d, *J* = 2.4 Hz) were assigned as H-3 and H-5, respectively. A set of isoprenyl unit at δ_H 2.31 (2H, m, H-1″), 4.92 (1H, t, *J* = 7.4 Hz, H-2″), and 1.56 (6H, s, 4″-CH_3 and 5″-CH_3). Two singlet resonance at δ_H 3.97 (3H, s) and 1.85 (3H, s) were assigned to a methoxy group attached at C-2 and methyl at C-3′, respectively. In addition, three methines at δ_H 4.57 (1H, d, H-1′), 3.48 (1H, m, H-6′), and 2.55 (1H, m, H-2′) were assigned as H-1′, H-6′, and H-2′, respectively. Therefore, structure **1** has a cyclohexenyl chalcone skeleton and was identified as isopanduratin A (Hwang et al., 2004).

Compound **2** was isolated as yellow solids. The ^{1}H NMR spectrum of compound **2** displayed characteristic resonances for an ABX spin system of a flavanone at δ_H 5.60 (1H, dd, *J* = 12.7, 3.1 Hz, H-2), 3.20 (1H, dd, *J* = 17.4, 12.7 Hz, H-3b) and 2.84 (1H, dd, *J* = 17.4, 3.1 Hz, H-3a), and a monosubstituted benzene ring at δ_H 7.39-7.59 (5H, m). A pair of meta-coupled doublets at δ_H 6.11 (1H, d, *J* = 2.1 Hz) and 6.08 (1H, d, *J* = 2.1 Hz) were assigned as H-6 and H-8, respectively. In addition, a methoxy group at δ_H 3.87 (3H, s) is attached at C-7. Thus, structure **2** was identified as pinostrobin (Smolarz et al., 2006). Analysis of the ^{1}H NMR data of compound **3** revealed it is an analog of compound **2**. However, the methoxy group in compound **3** disappeared. A comparison of the NMR data of compound **3** with those of pinocembrin (Maciejewicz, 2001) confirmed its structure.

Compound **4** was isolated as yellow solids. The ^{1}H NMR spectrum displayed a pair of trans-coupled protons at δ_H 8.01 (1H, t, *J* = 15.7 Hz) and 7.77 (1H, t, *J* = 15.7 Hz) indicating a chalcone scaffold. A strongly deshielded proton signal at δ_H 14.18 suggested an OH was attached at C-2′ and a monosubstituted benzene ring at δ_H 7.43-7.74 (5H, m). Two coupled doublet resonances at δ_H 6.03 (1H, d, *J* = 2.1 Hz) and 6.10 (1H, d, *J* = 2.1 Hz) were assigned as H-3′ and H-5′, respectively. A singlet at δ_H 3.99 (3H, s) was a methoxy group attached at C-6′. From above data, compound **4** is chalcone and was identified as cardamonin (He et al., 2005). Analysis of the ^{1}H NMR spectrum of compound **5** revealed that it is structurally related to compound **4**. However, the methoxy group in compound **5** was assigned to C-4′. A comparison of **5** with literature spectral data with those of pinostrobin chalcone (Malek et al., 2011) confirmed its structure.

4.2 Antimicrobial Activity

B. rotunda rhizome extract and fractions (A-D) were tested using the Kirby-Bauer disk diffusion assay for antimicrobial activity. It is a simple and relatively rapid primary screen. Ethyl acetate crude extract, fractions A and D (Table 1) gave antimicrobial activity against MRSA USA-300 LAC. Based on these results, fractions containing pinocembrin, pinostrobin chalcone, isopanduratin A exhibited significant antimicrobial activity with the zone of inhibition in a range between 12-15 mm (Table 1). Ampicillin and dimethyl sulfoxide (DMSO) are positive and negative controls for the experiment. The isolated compounds were then subject to broth microdilution assay to determine their minimum inhibitory concentrations.

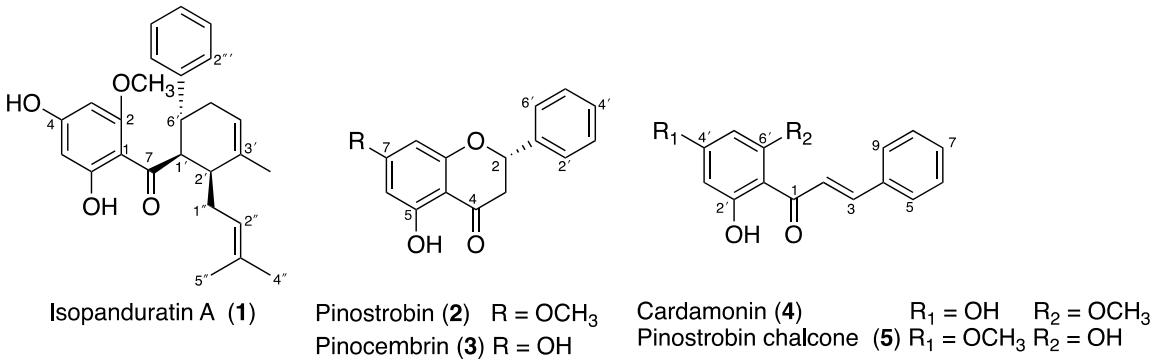


Figure 1 Structures of compounds isolated from *B. rotunda* rhizome extract

Table 1 Inhibition zone of samples from *B. rotunda* rhizome extract on MRSA USA-300 LAC

Sample	Inhibition Zone (mm)
Isopanduratin A (**1**)	15
Pinostrobin (**2**)	Inactive
Pinocembrin (**3**)	12
Cardamonin (**4**)	Inactive
Pinostrobin chalcone (**5**)	12
Fraction A	14
Fraction D	12
EtOAc extract	11
Ampicillin	26
DMSO	0

All isolated compounds obtained were evaluated for their antimicrobial activity minimum inhibitory concentrations (MICs) against nine Gram-positive (MRSA USA-300 LAC, MSSA HBP10, MRSA N315, MSSA LUU7, MSSA ONE6, MSSA R113, *S. aureus* NEWMAN, *S. aureus* 8325-4, and *S. aureus* ONE9) and two Gram-negative (*E. coli* and *S. marcescens*) bacteria, as summarized in Table 2. MRSA USA-300 LAC is known globally for its resistance to both beta-lactams and non-beta lactams, with its virulence directly affected by a mutation of the *sar*Z gene (Enström et al., 2018). A few strains isolated from Hawai'i were used in this study. For example, HBP10 is a strain of MSSA isolated from Hawai'i that carries the genes erm(C), aph(3')-III, and *mecA*, providing it resistance to macrolide, aminoglycoside, and beta lactam antibiotics, respectively (Gerken et al., 2021). MSSA LUU7 is a strain that contains the *bla*Z gene (beta-lactam resistance); whereas ONE6 is known for its virulence factors (*hlgA, hlgB, hlgC, lukD, and lukE*).

The results of this study demonstrated the broad-spectrum antimicrobial activity of *B. rotunda* rhizome ethyl acetate extract and isolated compounds (specifically isopanduratin A) against the prevalence of antimicrobial resistance (AMR) in clinical strains and Hawaiian strains of AMR *S. aureus* (Table 2).

Isopanduratin A showed potent antimicrobial activity against Gram-positive bacteria with MIC values ranging from 2 to 4 μg/mL, as shown in Table 2. Isopanduratin A also showed moderate activity against *S. marcescens* (Gram-negative bacteria) with a MIC value of 32 μg/mL. All remaining compounds showed either weak (MIC 64−128 μg/mL) or no perceptible antimicrobial activity against all tested bacterial strains. Vancomycin and gentamicin were positive controls for Gram-positive and Gram-negative bacteria, respectively. Their concentrations in inhibiting bacterial growth was between 0.5 and 0.25 μg/mL.

Table 2 MIC values (μg/mL) of samples from *B. rotunda* rhizome extract

Sample	MIC (μg/mL)										
	Gram-positive									Gram-negative	
	MRSA USA-300	**MRSA HBP10**	**MRSA N315**	**MSSA LUU7**	**MSSA ONE6**	**MSSA R113**	***S. aureus* Newman**	***S. aureus* 8325-4**	***S. aureus* ONE9**	***E. coli***	***S. marcescens***
Isopanduratin A (**1**)	4	2	4	4	2	2	4	4	2	128	32
Pinostrobin (**2**)	>128	128	>128	128	128	128	>128	>128	>128	128	64
Pinocembrin (**3**)	>128	64	128	128	64	64	>128	128	128	>128	64
Cardamonin (**4**)	>128	128	>128	>128	64	128	>128	>128	>128	128	64
Pinostrobin chalcone (**5**)	128	128	>128	128	128	64	>128	128	128	>128	64
EtOAc extract	320	20	640	320	320	20	640	320	640	>1280	640
Vancomycin	0.25	0.5	0.25	0.25	0.25	0.5	0.5	0.25	0.25	-	-
Gentamicin	-	-	-	-	-	-	-	-	-	0.25	0.25

4.3 Antioxidant Activity

Free radicals are chemical species containing at least one unpaired electron in a valence orbital (Pham-Huy et al., 2008). Free radicals are produced from the body's natural biological functions; yet, under chronic inflammation, the overproduction of reactive oxygen species could lead to oxidative stress, and free radicals can interact with proteins and nucleic acids within the body and may cause degenerative diseases such as cancer (Pham-Huy et al., 2008; Lobo et al., 2010). It is significant to search for natural antioxidants with high scavenging free radical capabilities from the body, decreasing oxidative stress.

Antioxidant properties of samples were determined using FRAP assay in which ferric ions are reduced to ferrous ions in the presence of an antioxidant that forms a blue-colored ferrous tripyridyltriazine complex (Fe^{2+}-TPTZ) (Pandey, & Rizvi, 2012). It is rapid, inexpensive, and characterized by overall sensitivity. In this study, *B. rotunda* ethyl acetate extract showed reductive capacity in reducing Fe^{3+} to Fe^{2+} in the FRAP assay. The flavonoids, the most prominent plant antioxidants, are a large class of phenolic compounds that act as free radical scavengers (Romanova et al., 2001). As presented in Table 3. The FRAP assay data show that flavonoids such as pinostrobin chalcone and cardamonin resulted in the highest antioxidant activity at 1 mg/mL with FRAP values of 17.76 ± 0.65 μM/μg, and 13.05 ± 0.23 μM/μg, respectively, compared with other compounds in this study. Isopanduratin A has high antioxidant activity at 1 mg/mL with FRAP value of 14.53 ± 0.11 μM/μg. Pinocembrin showed moderate antioxidant activity among compounds isolated, with a FRAP value of 8.74 ± 1.84 μM/μg at 1 mg/mL. For comparison, ascorbic acid (positive control) yielded a FRAP value of 27.92 ± 0.50 μM/μg. These results are comparable with the findings of literature that compounds containing more methoxy and phenolic hydroxyl groups exhibit more antioxidant activity (Chen et al., 2020). Furthermore, the *a*, *β*-unsaturated carbonyl group in cardamonin and pinostrobin chalcone may contribute to their antioxidant properties (Castaneda et al., 2017).

Table 3 FRAP Values of Samples (1 mg/mL) from *B. rotunda* (R^2 = 0.997)

Sample	FRAP Value (μM/μg)
Isopanduratin A (**1**)	14.53 ± 0.11
Pinostrobin (**2**)	9.03 ± 0.32
Pinocembrin (**3**)	8.74 ± 1.84
Cardamonin (**4**)	13.05 ± 0.23
Pinostrobin chalcone (**5**)	17.76 ± 0.65
EtOAc extract	2.05 ± 0.32
Ascorbic acid	27.92 ± 0.50

Antioxidant properties of samples suggested that phenolic groups at either *ortho* or *para*

positions of an aromatic ring may contribute to antioxidant activity, explaining why pinostrobin chalcone and cardamonin demonstrated the highest activity (Sökmen, & Akram Khan, 2016). Overall, the higher FRAP values for pinostrobin chalcone, isopanduratin A, and cardamonin can be attributed to the presence of more methoxy and hydroxyl functional groups in their structures and the position of these groups, resulting in more potent free-radical scavenging activity in some compounds than others.

4.4 Cytotoxic Activity

Cancer is a complex chronic condition characterized by anomalous signaling biochemical processes resulting in aberrant cellular growth (Ling et al., 2019). Nature is a valuable source of diverse molecules with unique chemical structures that can provide cytotoxic potential in various cancer cells and tumor entities (Newman et al., 2012). SRB assay is the preferred high-throughput assay by the National Cancer Institute (NCI) for the lead compound screening program (Van et al., 2015). The SRB assay utilizes sulforhodamine B that binds stoichiometrically to proteins in a slightly acidic condition (Orellana, & Kasinski, 2016). It can be detached when treated under basic condition (Zhao et al., 2022). Subsequently, the colorimetric evaluation of detached dye provides an estimated total protein mass related to cell number.

Cardamonin demonstrated the highest cytotoxic effect against the A549 lung cancer cell line, with an IC_{50} of 19.43 ± 0.33 μM that was significantly ($p<0.001$) lower than the other compounds except isopanduratin A, which exhibited a comparable effect ($p>0.05$) (Figure 3). Cardamonin lowered the viability significantly ($p<0.001$) at as low as 1.25 μg/ml concentration when compared with the control as well as all the other compounds isolated from *B. rotunda* (Figure 2). Cardamonin, a naturally occurring chalcone, has been utilized for numerous ailments in South America (Ramchandani et al, 2020). These results coincide with a previous study, where cardamonin induces apoptosis by activating caspase-3, upregulating Bax, and downregulating Bcl-2 in A549 (Ramchandani et al, 2020). In addition, cardamonin arrested cells in the G2/M phase and inhibited the expression levels of cyclin D1/CDK4. These effects were correlated with decreased phosphorylation levels of the downstream effectors of phosphoinositide 3-kinase (PI3K), including protein kinase-B (Akt/PKB) and mammalian target of rapamycin (mTOR) (Zhou et al., 2019).

Interestingly, with the same molecular formula, pinostrobin chalcone (only differing in the position of 6'-hydroxy and 4'-methoxy groups) showed a substantial decrease in effectiveness against A549 in the SRB assay, with six-fold IC_{50} value (115.59 ± 16.55 μM) compared to that of cardamonin (Malek et al., 2011). This shows the imperative role of the position of 6'-hydroxy and 4'-methoxy substituents in terms of cytotoxic effects.

Isopanduratin A also exhibited potential cytotoxicity against A549 with IC_{50} of 26.84 ± 1.06 μM. However, the mechanism of action of isopanduratin A's cytotoxic effects on A549 have yet to be studied. It induced caspase 3/7 in hepatocellular carcinoma cells (HepG2) (Nguyen et al., 2020).

Among other isolated compounds, pinostrobin and pinocembrin exhibited moderate cytotoxicity against A549 (IC_{50} of 93.62 ± 9.70 and 141.22 ± 5.85 μM, respectively), which is in agreement with a prior investigation where these compounds showed cytotoxicity against murine leukemia P-388 cells (Tanjung et al., 2013). The extract of *B. rotunda* contains several compounds with significant cytotoxic effects that inhibit A549 cancer cell proliferation.

Table 4 Cytotoxicity of *B. rotunda* samples against A549 cells. IC_{50} values were determined by SRB cytotoxicity assay on A549 cells treated with extract, isolated compounds or cisplatin for 72 h at a concentration ranging from 1.25 to 50 μg/mL (for extract and compounds) or 0.5 to 20 μM (for cisplatin). Results are shown as the mean ± standard deviation values of three independent experiments.

Sample	IC_{50} (μg/mL)	IC_{50} (μM)
Isopanduratin A (**1**)	10.91 ± 0.43	26.84 ± 1.06
Pinostrobin (**2**)	25.30 ± 2.62	93.62 ± 9.70
Pinocembrin (**3**)	36.19 ± 1.50	141.22 ± 5.85
Cardamonin (**4**)	5.25 ± 0.09	19.43 ± 0.33
Pinostrobin chalcone (**5**)	31.21 ± 4.47	115.59 ± 16.55
EtOAc extract	22.59 ± 0.16	N/A*
Cisplatin	1.00±0.009	3.33 ± 0.03

**EtOAc extract is a mixture with undetermined molar concentration*

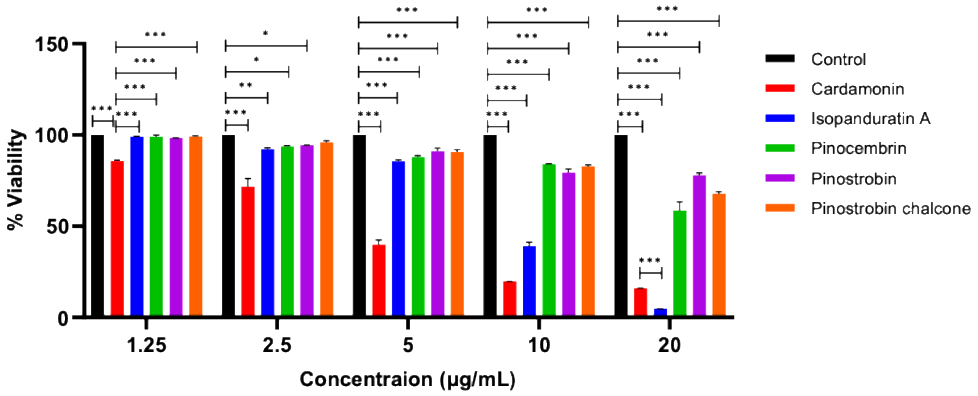


Figure 2 Cytotoxicity evaluation of compounds isolated from *B. rotunda* with the SRB assay. A549 cells were treated with different concentrations (1.25, 2.5, 5, 10, or 20 µg/mL) of each compound for 72 h. Results are given in viability percentages related to untreated control cells. Results are shown as the mean ± standard deviation values of three independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.001 between two groups indicated.

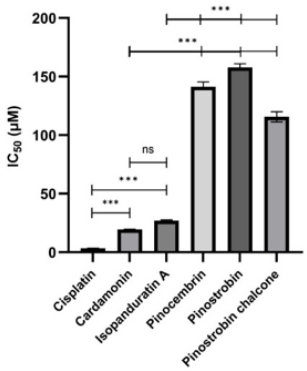


Figure 3 IC_{50} values of compounds and cisplatin towards A549 cells assessed via SRB assay. The cells were treated with samples for 72 hours at an increasing concentration. Results are shown as the mean ± standard deviation values of three independent experiments. ns= not significant, *p < 0.05, **p < 0.01 and ***p < 0.001 between two groups indicated.

6. Conclusion

Five compounds were isolated from the rhizomes of *B. rotunda*. Among the isolates, isopanduratin A (**1**) demonstrated the greatest potential as antimicrobial properties on both clinical strains and Hawaiian strains of methicillin-resistant *Staphylococcus aureus* and methicillin-susceptible *S. aureus*, with MIC values between 4 µg/mL to 2 µg/mL. Pinostrobin chalcone, isopanduratin A, and cardamonin showed the highest antioxidant activity (17.76 ± 0.65 µM/µg, 14.53 ± 0.11 µM/µg, and 13.05 ± 0.23 µM/µg, respectively) based on a FRAP assay due to the presence of phenolic hydroxyl groups in their structures. Compounds from *B. rotunda* rhizomes such as isopanduratin A (**1**) and cardamonin (**4**) showed effectiveness against A549 lung cancer cells (IC_{50} of 26.84 ± 1.06 and 19.43 ± 0.33 µM, respectively). The results of this study warrant further investigation into the isolation of secondary metabolites from *B. rotunda* rhizomes and their potential for pharmaceutical applications.

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8. References

Adalbert, J. R., Varshney, K., Tobin, R., & Pajaro, R. (2021). Clinical outcomes in patients co-infected with COVID-19 and *Staphylococcus aureus*: a scoping review. *BMC infectious diseases*, *21*(1), 1-17. http://doi.org/10.1186/s12879-021-06616-4

Baharudin, M. K. A., Hamid, S. A., & Susanti, D. (2015). Chemical composition and antibacterial activity of essential oils from three aromatic plants of the Zingiberaceae family in Malaysia. *Journal of Physical Science*, *26*(1), 71-81.

Bauer, A. W., Kirby, W. M. M., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, *45*(4), 493-496. http://doi.org/10.1093/ajcp/45.4_ts.493

Boucher, H. W., & Corey, G. R. (2008). Epidemiology of methicillin-resistant *Staphylococcus aureus*. *Clinical Infectious Diseases*, *46*(5), S344–S349. http://doi.org/10.1086/533590

Castaneda, O. A., Lee, S. C., Ho, C. T., & Huang, T. C. (2017). Macrophages in oxidative stress and models to evaluate the antioxidant function of dietary natural compounds. *Journal of Food and Drug Analysis*, *25*(1), 111-118. http://doi.org/10.1016/j.jfda.2016.11.006

Chen, C. J., & Huang, Y. C. (2014). New epidemiology of *Staphylococcus aureus* infection in Asia. *Clinical Microbiology and Infection*, *20*(7), 605-623. http://doi.org/10.1111/1469-0691.12705

Chen, J., Yang, J., Ma, L., Li, J., Shahzad, N., & Kim, C. K. (2020). Structure-antioxidant activity relationship of methoxy, phenolic hydroxyl, and carboxylic acid groups of phenolic acids. *Scientific Reports*, *10*(1), Article 2611. http://doi.org/10.1038/s41598-020-59451-z

Elshikh, M., Ahmed, S., Funston, S., Dunlop, P., McGaw, M., Marchant, R., & Banat, I. M. (2016). Resazurin-based 96-well plate microdilution method for the determination of minimum inhibitory concentration of biosurfactants. *Biotechnology Letters*, *38*, 1015-1019. http://doi.org/10.1007/s10529-016-2079-2

Eng-Chong, T., Yean-Kee, L., Chin-Fei, C., Choon-Han, H., Sher-Ming, W., Li-Ping, C. T., ... & Yusof, R. (2012). *Boesenbergia rotunda*: from ethnomedicine to drug discovery. *Evidence-Based Complementary and Alternative Medicine, 2012*, Article 473637. http://doi.org/10.1155/2012/473637

Enström, J., Fröding, I., Giske, C. G., Ininbergs, K., Bai, X., Sandh, G., ... & Fang, H. (2018). USA300 methicillin-resistant *Staphylococcus aureus* in Stockholm, Sweden, from 2008 to 2016. *PLoS One*, *13*(11), Article e0205761. http://doi.org/10.1371/journal.pone.0205761

Fukunaga, B. T., Sumida, W. K., Taira, D. A., Davis, J. W., & Seto, T. B. (2016). Hospital-acquired methicillin-resistant *Staphylococcus aureus* bacteremia related to medicare antibiotic prescriptions: A state-level analysis. *Hawai'i Journal of Medicine & Public Health*, *75*(10), 303-309.

Gerken, T. J., Roberts, M. C., Dykema, P., Melly, G., Lucas, D., De Los Santos, V., Gonzalez, J., Butaye, P., & Wiegner, T. N. (2021). Environmental Surveillance and Characterization of Antibiotic Resistant *Staphylococcus aureus* at Coastal Beaches and Rivers on the Island of Hawai'i. *Antibiotics*, *10*(8), Article 980. https://doi.org/10.3390/antibiotics10080980

He, W., Li, Y., Liu, J., Hu, Z., & Chen, X. (2005). Specific interaction of chalcone-protein: Cardamonin binding site II on the human serum albumin molecule. *Biopolymers: Original Research on Biomolecules*, *79*(1), 48-57. http://doi.org/10.1002/bip.20328

Hudzicki, J. (2009). Kirby-Bauer disk diffusion susceptibility test protocol. *American Society for Microbiology*, *15*, 55-63.

Hwang, J. K., Chung, J. Y., Baek, N. I., & Park, J. H. (2004). Isopanduratin A from *Kaempferia pandurata* as an active antibacterial agent against cariogenic *Streptococcus mutans*. *International Journal of Antimicrobial Agents*, *23*(4), 377-381. http://doi.org/10.1016/j.ijantimicag.2003.08.011

Islam, M. A., Atanu, M. S. H., Siraj, M. A., Acharyya, R. N., Ahmed, K. S., Dev, S., ... & Das, A. K. (2023). Supplementation of

syringic acid-rich *Phrynium pubinerve* leaves imparts protection against allergic inflammatory responses by downregulating iNOS, COX-2, and NF-κB expressions. *Heliyon*, *1*, 9(2), Article e13343. https://doi.org/10.1016/j.heliyon.2023.e13343

Jing, L. J., Mohamed, M., Rahmat, A., & Bakar, M. F. A. (2010). Phytochemicals, antioxidant properties and anticancer investigations of the different parts of several gingers species (*Boesenbergia rotunda, Boesenbergia pulchella var attenuata and Boesenbergia armeniaca*). *Journal of Medicinal Plants Research*, *4*(1), 27-32. http://doi.org/10.5897/JMPR09.308

Ling, T., Lang, W. H., Maier, J., Quintana, C. M., & Rivas, F. (2019). Cytostatic and cytotoxic natural products against cancer cell models. Molecules, *24*(10), Article 2012. https://doi.org/10.3390/molecules24102012

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews*, *4*(8), 118-126. http://doi.org/10.4103/0973-7847.70902

Maciejewicz, W. (2001). Isolation of flavonoid aglycones from propolis by a column chromatography method and their identification by GC-MS and TLC methods. *Journal of Liquid Chromatography & Related Technologies*, *24*(8), 1171-1179. http://doi.org/10.1081/JLC-100103439

Mahesh, B., & Satish, S. (2008). Antimicrobial Activity of Some Important Medicinal Plant against Plant and Human Pathogens. *World Journal of Agricultural Research*, *4*(5), 839-843.

Malek, S. N. A., Phang, C. W., Ibrahim, H., Wahab, N. A., & Sim, K. S. (2011). Phytochemical and cytotoxic investigations of *Alpinia mutica* Rhizomes. *Molecules*, *16*(1), 583-589. http://doi.org/10.3390/molecules16010583

Newman, D. J., & Cragg, G. M. (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of natural products*, *75*(3), 311-335. http://doi.org/ 10.1021/np200906s

Nguyen, S. T., Do, N. M., Tran, D. H., To, N. B., Vo, P. H., Nguyen, M. T. T., ... & Pham, P. V. (2020). Isopanduratin A Isolated from *Boesenbergia pandurata* Reduces HepG2 Hepatocellular Carcinoma Cell Proliferation in Both Monolayer and Three-Dimensional Cultures. *Advances in Experimental Medicine and Biology*, *1292*, 131–143. http://doi.org/10.1007/5584_2020_523

Ongwisespaiboon, O., & Jiraungkoorskul, W. (2017). Fingerroot, *Boesenbergia rotunda* and its aphrodisiac activity. *Pharmacognosy Reviews*, *11*(21), 27-30. http://doi.org/10.4103/phrev.phrev_50_16

Orellana, E. A., & Kasinski, A. L. (2016). Sulforhodamine B (SRB) assay in cell culture to investigate cell proliferation. *Bio-protocol*, *6*(21), Article e1984. http://doi.org/10.21769/BioProtoc.1984

Pandey, K. B., & Rizvi, S. I. (2012). Ferric reducing and radical scavenging activities of selected important polyphenols present in foods. *International Journal of Food Properties*, *15*(3), 702-708. http://doi.org/10.1080/10942912.2010.498547

Pham-Huy, L. A., He, H., & Pham-Huy, C. (2008). Free Radicals, Antioxidants in Disease and Health. *International Journal of Biomedical Science*, *4*(2), 89-96.

Ramchandani, S., Naz, I., Dhudha, N., & Garg, M. (2020). An overview of the potential anticancer properties of cardamonin. *Exploration of Targeted Anti-tumor Therapy*, *1*(6), 413-426. http://doi.org/10.37349/etat.2020.00026

Rayner, C., & Munckhof, W. J. (2005). Antibiotics currently used in the treatment of infections caused by *Staphylococcus aureus*. *Internal Medicine Journal*, *35*, S3-S16. http://doi.org/10.1111/j.1444-0903.2005.00976.x

Reller, L. B., Weinstein, M., Jorgensen, J. H., & Ferraro, M. J. (2009). Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clinical Infectious Diseases*, *49*(11), 1749-1755. http://doi.org/10.1086/647952

Romanova, D., Vachalkova, A., Cipak, L., Ovesna, Z., & Rauko, P. (2001). Study of antioxidant effect of apigenin, luteolin and quercetin by DNA protective method. *Neoplasma*, *48*(2), 104-107.

Smolarz, H. D., Mendyk, E., Bogucka-Kocka, A., & Kockic, J. (2006). Pinostrobin–an anti-leukemic flavonoid from *Polygonum lapathifolium* L. ssp. *nodosum* (Pers.) Dans. *Zeitschrift für Naturforschung C*, *61*(1-2), 64-68. http://doi.org/10.1515/znc-2006-1-212

Sökmen, M., & Akram Khan, M. (2016). The antioxidant activity of some curcuminoids and chalcones. *Inflammopharmacology*, *24*(2-3), 81-86. http://doi.org/10.1007/s10787-016-0264-5

Tanjung, M., Tjahjandarie, T. S., & Sentosa, M. H. (2013). Antioxidant and cytotoxic agent from the rhizomes of *Kaempferia pandurata*. *Asian Pacific Journal of Tropical Disease*, *3*(5), 401-404. http://doi.org/10.1016/S2222-1808(13)60091-2

Tang, S. W., Sukari, M. A., & Ee, G. C. L. (2007). Characterization of flavonoid derivatives from *Boesenbergia rotunda* (L.). Malay*sian Journal of Analytical Sciences, 11*(1), 154-159.

Van, T. A., Joubert, A. M., & Cromarty, A. D. (2015). Limitations of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. *BMC research notes*, *8*, 1-10. http://doi.org/10.1186/s13104-015-1000-8

Yusuf, N. A., M Annuar, M. S., & Khalid, N. (2013). Existence of bioactive flavonoids in rhizomes and plant cell cultures of *Boesenbergia rotunda* (L.) Mansf. Kulturpfl. *Australian Journal of Crop Science*, *7*(6), 730-734.

Zhao, C. L., Chik, W. I., & Zhang, H. J. (2022). Bioprospecting and bioassay-guided isolation of medicinal plants—A tool for drug discovery. In *Evidence-Based Validation of Herbal Medicine* (pp. 511-537). Amsterdam: Elsevier.

Zhou, X., Zhou, R., Li, Q., Jie, X., Hong, J., Zong, Y., ... & Wu, G. (2019). Cardamonin inhibits the proliferation and metastasis of non-small-cell lung cancer cells by suppressing the PI3K/Akt/mTOR pathway. *Anti-Cancer Drugs*, *30*(3), 241-250. http://doi.org/10.1097/CAD.0000000000000709