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Antioxidant capacity, antibacterial activity, and cell cytotoxicity in cholangiocarcinoma (CCA) from *Boesenbergia rotunda* (L.) Mansf.

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Abstract

Boesenbergia rotunda (L.) Mansf. rhizome was collected during different seasons and cultivation areas, defined as S1, S2, and S3. The rhizome extract was prepared by sequential maceration in hexane, ethyl acetate, and methanol. The rhizome extracts were evaluated for antioxidant, total phenolic and flavonoid content, cytotoxicity against cholangiocarcinoma (CCA), and pathogenic bacteria effects. The S2 extract in hexane and ethyl acetate demonstrated the highest Trolox Equivalent Antioxidant Capacity (TEAC) at 2.650 ± 0.215 and 2.089 ± 0.405, respectively. The highest total phenolic and flavonoid content were found in the S1 and S3 ethyl acetate extracts at 18.187 ± 0.961 mg- Gallic acid Equivalent (GE)/g-Dry Material (DM) and 381.863 ± 18.917 mg-Quercetin Equivalents (QE)/100 g-DM, respectively. The greatest cytotoxicity against CCA was found in the S2 ethyl acetate extract with an IC_{50} value of 22.65 ± 0.68 μg/mL, however, this did not affect normal cholangiocytes. Moreover, the cytotoxic effect on CCA may have involved apoptosis based on the chromatin condensation and nuclear fragment seen using fluorescence microscopy with 4′,6-diamidino-2-phenylindole staining. The S1 ethyl acetate extract was cytotoxic to gram-positive bacteria. The inhibition zone was 10.5, 9.5, 8.5, and 11.8 mm for *Staphylococcus aureus* ATCC 25923, Methicillin-resistant *S. aureus* (MRSA), *S. epidermidis*, and *Bacillus cereus*, respectively. In conclusion, this study demonstrated that there are active components in *B. rotunda* rhizome hexane and ethyl acetate extracts that have antioxidant, anticancer, and antibacterial activities. The active ingredients should be characterized in future studies.

Keywords: Antioxidant capacity, *Boesenbergia rotunda*, Cholangiocarcinoma, Antibacterial activity

1. Introduction

Boesenbergia rotunda (L.) Mansf. is classified as a ginger species, belonging to the *Boesenbergia* genus and Zingiberaceae family. This plant is widely distributed in Southeast Asia and China. It has been commonly used as a food product or traditional medicine [1,2]. The *B. rotunda* rhizome has been used in traditional medicine to treat inflammatory diseases (e.g. dental cavities, dermatitis, dry cough, and colds [3,4]), peptic ulcer from *Helicobacter pylori* infection [5], and to eliminate helminths in the human intestine [6]. The bioactive components in *B. rotunda* rhizome contain many plant secondary metabolites, such as flavonoid derivatives, essential oils, and polyphenolic compounds [7-11]. The phytochemical agents in *B. rotunda* rhizome have been investigated and the preparation method via extraction in solvents with different polarities has been reported. Sri A et al. have evaluated the substances in *B. rotunda* rhizome after ethanol extraction and solvent partitioning in hexane, chloroform, and ethyl acetate sequentially. Their results indicated that the active substances in the ethanol extract inhibited the growth of the human pathogens *Escherichia coli* strain ATCC 11229, *Staphylococcus aureus* strain ATCC 25923, *S. epidermidis* strain FNCC-0048, and *Streptococcus mutans* [12].

Moreover, the ethanol extract demonstrated wound-healing activity using a linear incision wound model in Sprague Dawley Rats [13]. The methanol extract from *B. rotunda* rhizome demonstrated anticandida activity

against *Candida albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* [14]. Furthermore, the active compound in the methanol extract, Pinostrobin, significantly and dose-dependently protected the gastric mucosa in an ethanol-induced injury model in Sprague Dawley Rats [15]. Anti-amoebic activity was found in *B. rotunda* rhizome extracts though the sequential maceration in chloroform, methanol and water. The chloroform extract inhibited cell viability in the trophozoites stage in *Entamoeba histolytica*, a parasite that is an opportunistic pathogen in patients with HIV infection [16,17]. Ongoing research in the cancer field is evaluating natural plant metabolites as potential targets for anticancer drug development. Kirana et al. found that *B. rotunda* rhizome extract is cytotoxic to HT-29 human colon cancer and MCF-7 human breast cancer cells [18]. Moreover, Jing and colleagues have revealed the cytotoxicity of rhizome extracts against CAOV-3 (human ovarian cancer), MDA-MB-231 (human breast cancer), and HeLa (cervical cancer cells) [11].

The aims of the present study were to examine the biochemical properties of *B. rotunda* rhizome extracts. We prepared the rhizome extracts by sequential maceration in solvents with different polarities; hexane, ethyl acetate, and methanol. The antioxidant capacity in each extract was determined via 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging methods. Total flavonoid and phenolic content were determined by a colorimetric technique using Folin-Ciocalteu and aluminium chloride reagents, respectively. The cytotoxicity of the rhizome extracts was determined using cholangiocarcinoma (CCA) cells that were assayed for cell viability and cell death via apoptosis using Methylthiazolyldiphenyltetrazolium bromide (MTT) and 4′,6-diamidino-2′-phenylindole dihydrochloride (DAPI) staining methods, respectively. We then evaluated the antibacterial activity of the rhizome extracts against the gram-positive and negative bacteria that cause human infectious diseases. Thus, the results obtained from this study should be useful for future investigations to identify new active compounds for pharmaceutical drug development.

2. Materials and methods

2.1 Sample preparation and extraction procedures

The plant taxonomy of *Boesenbergia rotunda* (L.) Mansf. was identified and compared with the voucher specimen deposited at the Plant Varieties Protection Office, Department of Agriculture, Bangkok, Thailand (Reference: BKK069788). *B. rotunda* rhizomes were purchased from a local market in Samut Prakan province, Thailand. The rhizome materials differed in the season cultivated, harvesting period, and cultivation areas, defined as S1, S2, and S3. The in-season cultivation for *B. rotunda* was between March-May. The plant is harvested after growing for 180 days. The S1 *B. rotunda* was bought in February (representing off-season cultivation). The S2 and S3 *B. rotunda* were bought in October (in-season cultivation), but from different cultivation areas. The rhizomes were cleaned and over-dried in an oven at 60 $^{\circ}$C for 3 days. The dry rhizomes were thoroughly blended and sequentially immersed in solvents with different polarities, hexane, ethyl acetate, and methanol. The maceration process was performed at a 1:4 (w/v) ratio of dry rhizomes (g) and extraction solvent (mL) at room temperature for 3 d. The liquid extract was filtered from the solid material using a filter paper (Whatman™ no.1). Subsequently, the solid material was repeatedly macerated in the same solvent three times and the pooled extracts had the excess solvent removed using a rotary evaporator (BUCHI Rotavapor® R-210, Switzerland). Finally, the extracts in each solvent were completely evaporated to remove the residual solvent using a speed vacuum concentrator (Savant SC-210A, Thermo Fisher Scientific, USA). The concentrated extracts were stored at -20 $^{\circ}$C until used.

2.2 Determination of antioxidant properties with ABTS and DPPH scavenging methods

2.2.1 ABTS scavenging method

The ABTS scavenging assay for measuring the antioxidant capacity of the *B. rotunda* rhizome extracts was slightly modified from Re et al. [19]. Briefly, ABTS radical cations ($ABTS^{\cdot+}$) were generated by mixing 10 mm ABTS solution and 2.45 mm potassium persulfate ($K_2S_2O_8$). The mixture was incubated in the dark at 4 $^{\circ}$C for 24 h before use. The extinction coefficient of the $ABTS^{\cdot+}$ stock solution was determined in 95% methanol at 734 nm based on the reported extinction coefficient in water [19]. The ABTS scavenging assay using 0.02–0.5 μg/mL ascorbate, 1.9-62.5 μg/mL BHT, and 0.93-15 μg/mL Trolox was performed to prepare a standard curve. The reaction mixture had a total volume of 1 mL containing 20 μl *B. rotunda* rhizome extracts or antioxidant standard reagents and 66 μM $ABTS^{\cdot+}$. The reaction occurred in the dark at room temperature for 10 min. The absorbance at 734 nm was collected by a UV-Vis spectrophotometer (GENESYS 10S Thermo Fisher Scientific, USA). The $ABTS^{\cdot+}$ solution and 95% methanol were used as control and blank, respectively.

2.2.2 DPPH scavenging method

The DPPH solution was freshly prepared in 95% methanol to determine the antioxidant activity of the *B. rotunda* rhizome extracts. The DPPH stock solution was calculated based on the extinction coefficient of 10,900 m/cm at 515 nm [20]. The concentration of the antioxidant standard agents for the DPPH scavenging assay were 0.2-6.25 μg/mL ascorbate, 1.9-62.5 μg/mL BHT, and 1.6-50 μg/mL Trolox. The reaction mixture had a total volume of 1 mL containing 20 μl of *B. rotunda* rhizome extracts or standard antioxidant agents and 0.1 mM DPPH. The mixture was incubated for 2 h at room temperature in the dark. The absorbance at 515 nm was collected by a UV-Vis spectrophotometer (GENESYS 10S Thermo Fisher Scientific, USA). DPPH free radicals and 95% methanol were used as control and blank, respectively. The DPPH and ABTS scavenging assay were calculated as inhibitory percentage using Equation 1.

$$\% \text{ inhibition} = 100 - [(Abs_0/Abs_1) \times 100\%] \quad (1)$$

Where Abs_0 and Abs_1 are the absorbance of the DPPH radical (515 nm) or $ABTS^{\cdot+}$ (734 nm) with or without an analytical sample (antioxidant agents or *B. rotunda* rhizome extracts), respectively.

The inhibitory percentage from Equation 1 was used to calculate the inhibitory concentration at 50% (IC_{50}). The experiment was performed using 6 concentrations in triplicate. The IC_{50} value was analyzed and fit using the non-linear sigmoidal curve equation in KALEIDAGRAPH (Synergy Software, Reading, PA, USA) according to Equation 2.

$$Y = m_1+(m_2-m_1)/[1+(x/m_3)\, m_4] \quad (2)$$

Where m_1 is the maximum value on the y-axis, m_2 is the minimum value on the y-axis, m_3 is the X value at mid-point of the Y range, and m_4 is the slope of the curve at the midpoint.

The antioxidant value was expressed as Trolox Equivalent Antioxidant Capacity (TEAC) based on Equation 3 [21]. The antioxidant standard agents, ascorbate and BHT, were compared with the antioxidant values of *B. rotunda* rhizome extracts in each extraction solvent.

$$\text{TEAC} = IC_{50} \text{ of Trolox}/IC_{50} \text{ of analytical sample} \quad (3)$$

2.3 Determination of the total phenolic and flavonoid content

2.3.1 Total phenolic content

The total phenolic content was determined with a colorimetric method using Folin-Ciocalteu reagent (Bio-Rad, USA). The quantitative method was slightly modified from Ainsworth EA and Gillespie KM [22]. Briefly, 0.2-8.5 μg/mL gallic acid (standard curve) was employed to determine the total phenolic content. The reaction assay contained 20 μl gallic acid or *B. rotunda* rhizome extracts that was homogenized with 10 μl Folin-Ciocalteu reagent. The reaction mixture was incubated at room temperature for 5 min. Next, 500 μl 1 M sodium carbonate (Na_2CO_3) was added to the mixture, the final volume was adjusted to 1 mL with 95% methanol, and incubated at room temperature for 2 h. The absorbance at 765 nm was collected by a UV-Vis spectrophotometer (GENESYS 10S Thermo Fisher Scientific, USA). Total phenolic content was expressed as mg Gallic Acid Equivalents per gram of Dry Material (mg-GAE/g-DM).

2.3.2 Total flavonoid content

Total flavonoid content was determined with a colorimetric method using aluminium chloride ($AlCl_3$) [23]. We used 4-40 μg/mL quercertin (standard curve) to determine the total flavonoid content. The reaction assay contained 20 μl quercetin or *B. rotunda* rhizome extracts that was homogenized with 30 μl 5% w/v sodium nitrite ($NaNO_2$) and incubated for 5 min. This mixture received 30 μl 10% w/v $AlCl_3$ and 200 μl of 1 M sodium hydroxide, and the final volume was adjusted to 1 mL with 95% methanol. The absorbance at 510 nm was collected by a UV-Vis spectrophotometer (GENESYS 10S Thermo Fisher Scientific, USA). The Total flavonoid content was expressed as mg Quercetin Equivalents per 100 g of Dry Material (mg-QE/100g-DM).

2.4 Cell viability assay using RMCCA-1 and MMNK-1 cells

The cell viability assay method was approved by the Ethics Committee of Huachiew Chalermprakiet University (Certificate of Exemption no. 651/2561). A human cholangiocarcinoma cell line, RMCCA-1, was established and kindly provided by Associate Professor Rutaiwan Tohtong, Faculty of Science, Mahidol

University, Thailand. To evaluate cell viability, the RMCCA-1 cells were cultured in HAM's F-12 media supplemented with 10% heat-inactivated fetal bovine serum (FBS) [24]. The MMNK-1 cells (JCRB Cell Bank no.1554) were cultured in DMEM supplemented with 5% heat-inactivated FBS. The cell culture media for the RMCCA-1 and MMNK-1 cells were supplemented with 15 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 μg/mL Streptomycin, 100 U/mL Penicillin G, and 0.25 μg/mL Amphotericin B. The cell cultures were maintained at 37 °C with 5% CO_2 in a humidified incubator. The RMCCA-1 and MMNK-1 cells were seeded in 96-well microplates at a density of 5,000 and 4,000 cells/well, respectively. The cell lines were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 24 h before treatment. The cells were treated with the *B. rotunda* rhizome extracts and the cytotoxic effect was compared with cisplatin and doxorubicin, common chemotherapy drugs in cancer research [25,26]. Cell viability was determined using an MTT assay. After a 48-h treatment, 5 mg/mL MTT solution was added and incubated at 37 °C with 5% CO_2 for 4 h. The MTT was converted to insoluble formazan crystals by the viable cells and the formazan crystals were solubilized in DMSO. Cell viability was based on the solubilized formazan intensity and the absorbance was measured at 540 nm by a Microplate reader Multiskan (Thermo Fisher Scientific, USA). The percentage of cell growth inhibition and inhibitory concentration at 50% (IC_{50}) were calculated compared with untreated cells. The experiments were performed 3 times.

2.5 DAPI staining

We investigated if the cell viability assay results of the RMCCA-1 cells treated with *B. rotunda* rhizome extracts was due to apoptosis. This assay was performed using DAPI staining following the manufacturer's protocol (Cell Signaling Technology, USA). Briefly, approximately 100,000 cells/well were seeded on a sterile coverslip and placed on a 35-mm plate. The RMCCA-1 cells were allowed to attach and incubate at 37 °C in a humidified atmosphere with 5% CO_2 for 24 h before treatment. The cells were treated with the *B. rotunda* rhizome extracts and incubated at 37 °C for 24 h. At the end of the incubation period, the cells were washed with phosphate buffer saline (PBS) pH 7.4, fixed in 4% paraformaldehyde at room temperature for 5 min and washed with 0.1% Triton X-100 in PBS. The cells were stained with 300 μl 1 μg/mL DAPI in the dark at room temperature for 15 min and washed with PBS. To visualize the stained cells, the cells were mounted on a slide using 30% glycerol diluted in PBS, and covered with a coverslip. The stained cells were observed using a fluorescent microscope (Olympus IX71, Olympus Corporation, Japan). Image processing was performed by Image-Pro software (Media Cybernetics, Inc., Silver Spring, USA). The extraction solvents (hexane, ethyl acetate, and methanol) served as negative controls.

2.6 Antibacterial activity using the agar well diffusion method

Growth inhibition of the gram-positive and gam-negative bacterial pathogens was performed using the agar well diffusion method modified from Rauha JP et al. [27]. Briefly, bacterial colonies on blood agar were selected and inoculated in an adjusted volume of 0.5 McFarland standard (10^8 CFU/mL) adjusted with sterile normal saline [28]. The cell suspension was applied on Mueller-Hinton Agar (MHA) using a sterile cotton swab, left at room temperature for 5 min and a well was made with a No.2 cork borer. The wells were filled with 50 μl 1 mg/mL of each rhizome solvent extract from *B. rotunda* to inhibit bacteria growth and then incubated at 35 °C for 18-24 h. The extraction solvent and an antibiotic (tetracycline) were used as negative and positive control, respectively. The diameter (in mm) of the clear zone in each well was defined as the inhibition zone. This experiment was performed 3 times.

2.7 Statistical analysis

The data are presented as means ± standard deviation of the 3 independent experiments. A P-value < 0.05 was considered significant by One-way ANOVA analysis. All statistical analyses were performed using SPSS (Statistical Packages for Social Sciences) version 15 software.

3. Results and discussion

3.1 Antioxidant capacity, and total phenolic and flavonoid content in the B. rotunda rhizomes extracts

The concentrated *B. rotunda* rhizome extracts in each extraction solvent are less soluble in water, PBS, and DMSO. This problem affected the experiments that used absorption spectrophotometry. The rhizome extracts in each extraction solvent were dissolved in methanol to increase their solubility during the experimental assays. The rhizome extracts were prepared from the three different production sources defined as S1, S2, and S3. These

rhizome extracts were evaluated for their antioxidant capacity, cell cytotoxicity, and antibacterial activity against human pathogens.

The potency of the standard antioxidant agents and rhizome extracts were evaluated in terms of TEAC compared with Trolox. Active antioxidant capacity was found in only the hexane and ethyl acetate extracts in the S1, S2, and S3 groups (Table 1). The results in Table 1 indicated that the most effective antioxidant capacity was found in the S2 hexane extract with a TEAC value of 2.650 ± 0.215 based on the ABTS scavenging method.

However, the TEAC values from the DPPH scavenging method in the S2 rhizome extract were slightly different between the hexane and ethyl acetate solvents. The total phenolic content was the highest in the S1 ethyl acetate extract at 18.187 ± 0.961 mg-GE/g-DM. The total flavonoid content was highest in the S3 ethyl acetate group (381.86 ±18.917 mg-QE/100g-DM).

Table 1 Antioxidant activity, total phenolic and flavonoid contents in the *B. rotunda* extracts.

Sample[$]	Extraction solvent	TEAC[#]		Total phenolic (mg-GE/g-DM)[A]	Total flavonoid (mg-QE/100g-DM)[B]
		ABTS	DPPH		
S1	Hexane	1.63 ± 0.255	0.178 ± 0.049	7.359 ± 0.882	355.113 ± 66.917
	Ethyl acetate	0.53 ± 0.023	0.103 ± 0.019	18.187 ± 0.961	209.279 ± 61.727
	Methanol[*]	0.229 ± 0.009	0.015 ± 0.002	0.140 ± 0.009	ND
S2	Hexane	2.650 ± 0.215	0.171 ± 0.036	0.085 ± 0.002	ND
	Ethyl acetate	2.089 ± 0.405	0.179 ± 0.053	12.419 ± 1.005	176.061 ± 6.374
	Methanol[*]	0.074 ± 0.006	0.007 ± 0.0004	0.100 ± 0.008	ND
S3	Hexane	0.059 ± 0.005	0.004 ± 0.0005	0.119 ± 0.010	ND
	Ethyl acetate	1.558 ± 0.337	0.089 ± 0.009	5.852 ± 0.361	381.863 ± 18.917
	Methanol[*]	0.359 ± 0.044	0.034 ± 0.008	1.249 ± 0.061	ND
Antioxidant standard agent					
Ascorbate		49.396 ± 3.036	5.757 ± 0.352		
BHT		1.483 ± 0.290	0.971 ± 0.044		

[$] *B. rotunda* rhizome extracts were prepared from the different production sources defined as S1, S2, and S3.
[#] ABTS and DPPH scavenging methods were expressed in Trolox Equivalent Antioxidant Capacity (TEAC).
[A] Total phenolic content was expressed in milligram (mg) of Gallic acid Equivalent per gram of Dry Material (mg-GE/g-DM).
[B] Total flavonoid content was expressed in milligram (mg) of Quercetin Equivalent per 100 grams of Dry Material (mg-QE/100g-DM).
[*] Antioxidant capacity in methanol shown the significantly different from hexane and ethyl acetate at $p < 0.05$.
Data is presented as mean ± standard deviation (SD) with n = 3.
ND is not detected at the concentration tested.

3.2 Cytotoxicity activity and apoptosis

The effect of the *B. rotunda* rhizome extracts on cholangiocarcinoma (RMCCA-1) cells was examined using a cell cytotoxicity assay compared with the standard cancer drugs cisplatin and doxorubicin. In the cell viability assay, cholangiocytes (MMNK-1) were used as a control. The RMCCA-1 and MMNK-1 cell lines were treated with 12.5-400 μg/mL of the rhizome extracts. The results demonstrated that the highest cytotoxicity occurred in the S1, S2, and S3 ethyl acetate extracts. However, the hexane and methanol rhizome extracts were not cytotoxic to the RMCCA-1 or MMNK-1 cells. When treating the RMCCA-1 cells, the S1, S2, and S3 ethyl acetate rhizome extract demonstrated an IC_{50} of 77.89 ± 12.26, 22.65 ± 0.86, and 84.34 ± 11.50 μg/mL, respectively (Table 2). In contrast, the ethyl acetate rhizome extract was not cytotoxic to the MMNK-1 cells. The IC_{50} of the ethyl acetate rhizome extract when treating the MMNK-1 cells was more than 400 μg/mL. The IC_{50} of the cisplatin treatment of the RMCCA-1 and MMNK-1 cell lines was 14.91 ± 4.04 and 0.91 ±0.13 μg/mL, respectively. Moreover, the IC_{50} of the doxorubicin treatment of the RMCCA-1 and MMNK-1 cell lines was 8.19 ± 0.16 and 0.44 ± 0.07 μg/mL, respectively (Table 2). Cisplatin and doxorubicin were cytotoxic to both RMCCA-1 and MMNK-1 cell lines, however, the ethyl acetate rhizome extract was cytotoxic only to the RMCCA-1 cells.

The cell viability assay results suggested that the active component in the *B. rotunda* ethyl acetate rhizome extract induced apoptosis in the RMCCA-1 cells. DAPI staining was used to evaluate this hypothesis. Because

the ethyl acetate extracts were the most cytotoxic to the RMCCA-1 cells (IC_{50} in Table 2), the S2 rhizome extracts were selected for the DAPI staining. The RMCCA-1 cells were treated with 100 µg/mL hexane, ethyl acetate and methanol extracts and the change in cell morphology was evaluated using fluorescence microscopy. Apoptosis was observed in the RMCCA-1 cells treated with the ethyl acetate rhizome extract (Figure 1B). The cell morphology demonstrated chromatin condensation and nuclear fragmentation, which occurs in apoptosis. The RMCCA-1 cells treated with the control (ethyl acetate) (Figure 1A,) presented round and intact nuclei. Moreover, the hexane and methanol rhizome extracts had no effect on RMCCA-1 cell morphology (Figure 1D and 1F), which was similar to that seen in the control group (Figure 1C and 1E).

Table 2 IC_{50} value of the *B. rotunda* rhizome ethyl acetate extracts against RMCCA-1 and MMNK-1 cells.

Cell line	IC_{50} (µg/mL)**				
	Ethyl acetate extract			Cisplatin	Doxorubicin
	S1	S2	S3		
RMCCA-1	77.89 ± 12.26	22.65 ± 0.68	84.34 ± 11.50	14.91 ± 4.04	0.91 ± 0.13
MMNK-1	> 400	> 400	> 400	8.19 ± 0.66	0.44 ± 0.07

Data is presented in average ± standard deviation (SD) with n = 3.
**MTT cytotoxicity assay test was expressed in IC_{50} (µg/mL).

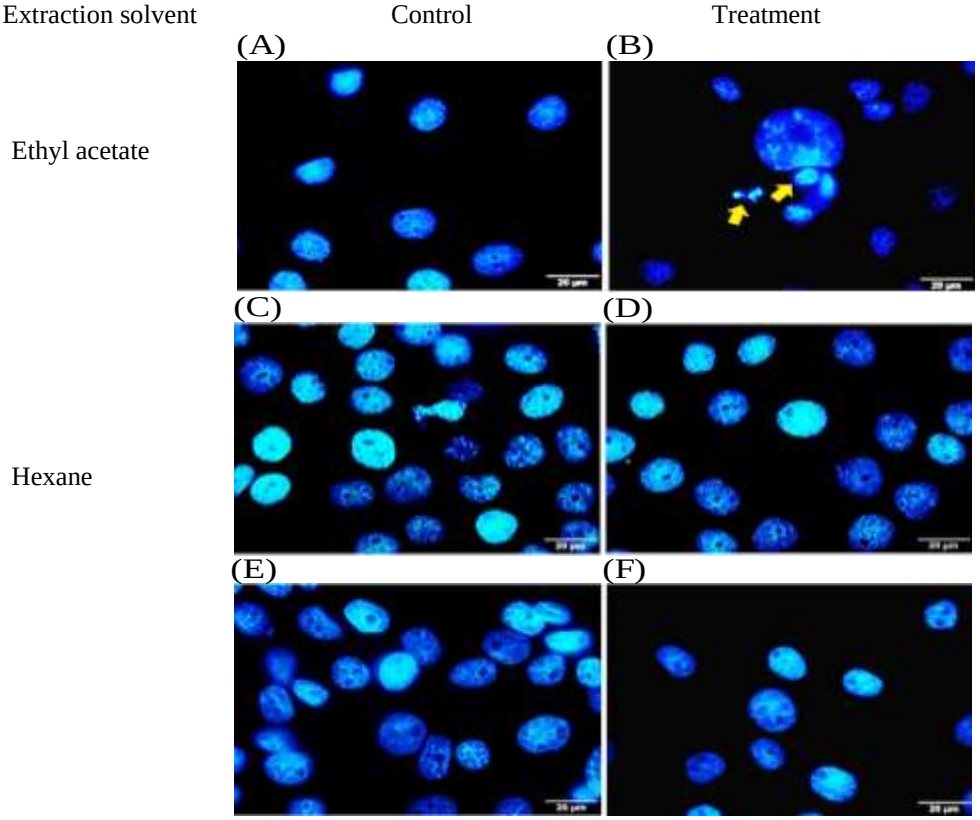

Figure 1 Fluorescence microscope image of the RMCCA-1 cells stained with DAPI.

Cells were treated with solvent as negative control compared with the S2 rhizome extracts. The stained cells in (1A) ethyl acetate, (1C) hexane, and (1E) methanol control. The stained cells in (1B) rhizome ethyl acetate extract, (1D) rhizome hexane extract, and (1F), as rhizome methanol extract. The yellow arrow in Figure (1B) indicates the changes in nuclear morphology, i.e. chromatin condensation and nuclear fragmentation.

3.3 Antibacterial activity

The rhizome extracts were evaluated for their antimicrobial activity against pathogenic gram-positive and gram-negative bacteria. The results revealed that the S1 ethyl acetate rhizome extracts were the most effective against the gram-positive bacteria evaluated (Table 3). The inhibition zone of the S1 ethyl acetate extract was 10.5, 9.5, 8.5, and 11.8 mm for *Staphylococcus aureus* (ATCC 25923), methicillin-resistant *S. aureus* (MRSA), *S. epidermidis*, and *Bacillus cereus*, respectively (Figure 2). The solvent control was used to

demonstrate the inhibition effect caused by the components of the ethyl acetate rhizome extract (Figure 3). The S1 hexane rhizome extract demonstrated antimicrobial activity against only the gram-positive bacteria, *Staphylococcus aureus* ATCC 25923, *S. epidermidis*, and *Bacillus cereus* (Table 3). Furthermore, the results demonstrated that the S1 hexane, ethyl acetate, and methanol extracts inhibited Bacillus cereus growth with an inhibition zone of 10, 11.8, and 9 mm, respectively.

Our results suggested that the different biochemical properties in each rhizome production group is due to the season in which it was cultivated, harvesting period, cultivars, and cultivation areas. The optimum temperature and humidity influence active component production, however, the mechanism of this remain unclear. The S1 (off-season cultivation), S2 and S3 (in-season cultivation, but from different cultivated areas) *B. rotunda* rhizome extracts have different antioxidant properties, total phenolic and flavonoid contents, and cytotoxicity to CCA and pathogenic bacteria. The S2 group was the most effective antioxidant (TEAC for the hexane and ethyl acetate extracts were 2.650 ± 0.215 and 2.089 ± 0.405, respectively). Moreover, the most effective cytotoxicity against CCA was present in the S1 ethyl acetate extract (IC_{50} value of 22.65 ± 0.68 μg/mL), however this was not cytotoxic to normal cholangiocytes). In contrast to the cytotoxic effect on CCA, the S1 (ethyl acetate extract) was cytotoxic to gram-positive pathogenic bacteria. The highest amounts of total phenolics and flavonoids were present in the S1 ethyl acetate extract with 18.187 ± 0.961 mg-GE/g-DM and S3 ethyl acetate extract with 381.863 ± 18.917 mg-QE/100g-DM, respectively.

Drug resistance is the main problem in cancer patients, leading to the failure of cancer medication treatment [29]. The current chemotherapeutic treatments for CCA patients still rely on using a single or combination of drugs [30]. The S1, S2, and S3 ethyl acetate rhizome extracts have components for treating CCA and do not affect normal cholangiocytes compared with cisplatin and doxorubicin treatment, which affect both. The most effective ethyl acetate rhizome extracts inhibited CCA proliferation, but not that of cholangiocytes (normal bile duct epithelial cells) proliferation based on IC_{50}. Treating RMCCA-1 cells with the S2 ethyl acetate extract resulted in nuclear fragmentation and small amounts of nucleus budding as demonstrated by DAPI staining. The microscopic cell morphology presented chromatin condensation and nuclear fragmentation, which is related to cell apoptosis. These results suggested that the cytotoxicity seen in CCA is due to apoptosis.

Antibacterial agents have revolutionized our ability to control bacterial diseases. However, antibiotic resistance is a challenge in public health. The most serious concern with antibiotic resistance is that some pathogenic bacteria have become resistant to almost all current antibiotics. It is estimated that by 2050, there will be no effective antibiotics available if no new drugs are developed or discovered [31]. This estimate indicates searching for new compounds as antibacterial agents is vital. In this study we found antibacterial activity in the S1 *B. rotunda* rhizome extracts. The ethyl acetate rhizome extract inhibited gram-positive bacterial, *S. aureus* ATCC 25923, methicillin-resistant *S. aureus* (MRSA), *S. epidermidis*, and *B. cereus*. However, the different cultivation season groups demonstrated different antibacterial activities. The active ingredients in the different cultivation season groups should be determined in future studies.

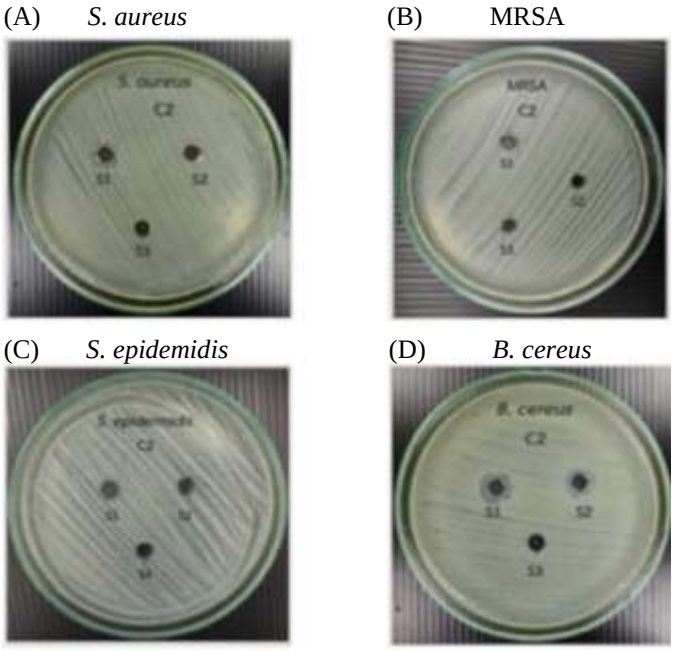

Figure 2 Antibacterial activity of the *B. rotunda* S1 and S2 rhizome ethyl acetate extracts against the gram-positive bacteria.

The S1 rhizome ethyl acetate extract (C2) generated an inhibition zone of 10.5, 9.5, 8.5, and 11.6 mm for *S. aureus* (Figure 2A), methicillin-resistant *S. aureus* (MRSA in Figure 2B), *S. epidermidis* (Figure 2C), and *B. cereus* (Figure 2D), respectively. The S2 rhizome ethyl acetate extract (C2) was only generated an inhibition zone for *B. cereus* (Figure 2D) of 10.5 mm. The agar well diffusion assay was performed on Mueller Hinton agar by spreading the tested bacteria and drilled to make a well. Fifty microliters of rhizome extract were filled into each well and then incubated at 35 $^{\circ}$C for 18-24 h.

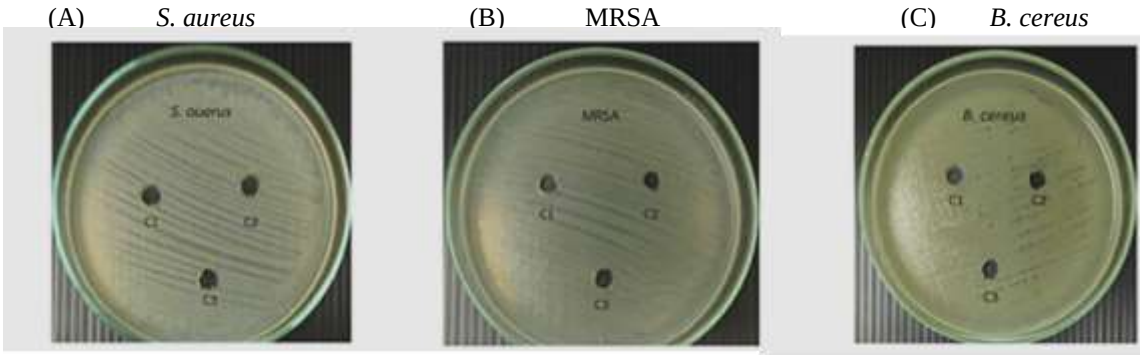

Figure 3 Antibacterial activity of the solvent control; C1, C2 and C3 was the negative control using hexane, ethyl acetate, and methanol, respectively.

The experiment was performed in Mueller Hinton agar using a well diffusion assay for *S. aureus* (Figure 3A), methicillin-resistant *S. aureus* (MRSA in Figure 3B), and *B. cereus* (Figure 3C).

Table 3 Antibacterial activity of the *B. rotunda* rhizome extracts in each solvent extraction against nine pathogenic bacteria.

Organism	Inhibition zone (mm)												
	Tetracycline	[#] Solvent control			*B. rotunda* rhizome extract								
		C1	C2	C3	C1			C2			C3		
					S1	S2	S3	S1	S2	S3	S1	S2	S3
Gram-positive bacteria													
Staphylococcus aureus ATCC 25923	31.0 ± 1.5[*]	NI	NI	NI	7.7 ± 0.5[*]	NI	NI	10.5 ± 2.3[*]	NI	NI	NI	NI	NI
Methicillin-resistant *S. aureus* (MRSA)	11.3 ± 1.4[*]	NI	NI	NI	NI	NI	NI	9.5 ± 1.9[*]	NI	NI	NI	NI	NI
S. epidermidis	34.0 ± 2.4[*]	NI	NI	NI	8.3[*]	NI	NI	8.5	NI	NI	NI	NI	NI
Bacillus cereus	34.7 ± 0.5[*]	NI	NI	NI	10.0 ± 1.5[*]	NI	NI	11.8 ± 1.6[*]	10.5	NI	9 ± 0.9	NI	NI
Gram negative bacteria													
Escherichia coli	25	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Vibrio parahaemolyticus	30	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Shigella spp.	30	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Acinetobacter baumannii	30	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Aeromonas hydrophila	16	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI

[#]The solvent was used in rhizome extraction defined as C1, C2, and C3 for hexane, ethyl acetate, and methanol, respectively.
[*]Data is presented in average $\pm$ standard deviation (SD) with n = 3
B. rotunda rhizome extracts were prepared from the different production sources defined as S1, S2 and S3. NI is defined as "No inhibition zone"

4. Conclusion

The present study has revealed that there are active components in the *B. rotunda* rhizome hexane and ethyl acetate extracts that have antioxidant, anticancer, and antibacterial activities. The amount of antioxidant activity, total phenolic, and flavonoid contents, cytotoxicity against CCA, and pathogenic bacteria were different

between the S1, S2, and S3 groups due to the cultivation season, harvesting period, and cultivated areas. The crude extracts demonstrated antioxidant properties, greatest content of total flavonoids, and cytotoxicity against CCA in the S2 and S3 (representing in-season cultivation) extracts, while an antibacterial effect was present in the S1 (out of season cultivation) extracts. Further investigations should focus on the identification and characterization of the active ingredients in the *B. rotunda* extracts to help develop promising drugs to treat cancer patients and pathogen infections in the future.

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