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Antibacterial activity of *Boesenbergia rotunda* (L.) Mansf. A. extract against *Escherichia coli*

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Abstract

An awareness of *Escherichia coli* as a foodborne pathogen and illness causing bacterium has been increased among consumers. Moreover, there is demand for natural product in order to reduce synthetic product that can cause toxic to the human. In this study, antibacterial activity, in term of MIC, MBC and killing-time curve of methanolic extract of *Boesenbergia rotunda* have been tested against a standard *E. coli* ATCC 25922 and two *E. coli* isolated from milk products using Clinical and Laboratory Standard Institute (CLSI) methods. The results show that *B. rotunda* extract was susceptible to all *E. coli* strains. The MIC and MBC values of *B. rotunda* extract against *E. coli* ranged 0.019 mg/mL 2.5 mg/mL and 0.039 mg/mL – 5.0 µg/mL, respectively. Killing-time curves were constructed at concentrations of 0x MIC, 1/2x MIC, 1x MIC, and 2x MIC. All *E. coli* strains can be killed with concentration of 2x MIC after 2 hours. The results show that *B. rotunda* extract has potential antibacterial activity against *E. coli.*

Introduction

Boesenbergia rotunda or *temukunci* has known as medicinal and culinary herb in Southeast Asia and it is widely use in Javanese cuisine. *B. rotunda* is belongs to a ginger (*Zingiberaceae*) in the order of Zingiberales and distributed from India to South East Asia. The medicinal plants are very useful in healing various diseases. It has been considered as a healthy source of life and percentage of people that used the medicinal plants as an alternative medicine has been grown in recent years. In fact there are more than 35,000 plants species that had been used as medicine and might be higher, that are passed orally by generation to generation (Lewington, 1993).

A previous study by Mahesh and Satish (2008) proved that the medicinal plants can be used as antimicrobial agents against human pathogens. It is supported by Iqbal *et al*. (1998) which found that the medicinal plants are rich source of antimicrobial agents and useful as powerful drugs. The active and non-bioactive compound in plants, such as phenolic compound, protein like compound, flavonoids and tannins plays an important role as antimicrobial agents. Kirana *et al.* (2007), found that *B. rotunda* contain essential oil, boesenbergin, cardamonin, pinostrobin, 5, 7-dimethoxyflavone, 1, 8-cineole, and also panduratin. *B. rotunda* extract has strong antibacterial activity against oral bacteria, enterococci and staphylococci (Rukayadi *et al.*, 2008; Rukayadi *et al.*, 2009; Yanti *et al*., 2009). Unfortunately, the antibacterial activity of *B. rotunda* extract against foodborne pathogens such as *E. coli* have not much been published yet.

New York Department of Health reported that *E. coli* is the common bacteria that live in humans and animals intestines. Several strains of these bacteria can cause diarrhea and infection of *E. coli* O157:H7 will cause severe diarrhea and also kidney damage. It can infect a person at any age but children and elderly are more likely to develop serious complications. This bacterium are acquired by eating food that is contaminated and infection can occur after consuming undercooked meat, lettuce, unpasteurized milk, juice or cider. Transmission by person-to-person can occur if the infected people do not wash their hands after using the toilet. Most outbreaks were caused by pathogenic bacteria especially *E. coli* O157:H7 and it can cause infection at dosage as low as 10-100 cfu/g (Mccarthy *et al*., 1998; Shearer *et al*., 2001). *E. coli* infection has become serious problem all over the world since this bacteria has develop the gene which can resist antibiotics that are frequently used and available in the market (Alonso *et al.*, 2000). Thus, the aim of this study is to determine the antibacterial activity of *B. rotunda* extract against *E. coli.*

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Materials and Methods

Extract preparation

The dried plant material, the rhizome of *B. rotunda* was ground and extracted with 400 mL of 100% (v/v) methanol for seven days at room temperature as stated by Rukayadi *et al.* (2008), with some modification. After seven days, the plant material was filtered using Whatman filter paper no. 2 and concentrated by using rotatory evaporator (50°C, 150 rpm) at Biochemical Laboratory, Faculty of Food Science and Technology, Universiti Putra Malaysia, Serdang. The extract was dissolved in 10% dimethylsulfoxide (DMSO) to obtain stock solution. The final concentration of extract was standardized at 10 mg/mL or 1%. 10% DMSO did not kill bacteria that being tested in this study.

Bacterial strains

E. coli ATCC 25922 was obtained from the American Type Culture Collection (Rockville, MD). Two isolates *E. coli* O157:H7, stated *E. coli* O157:H7-1 and *E. coli* O157:H7-2, were obtained from Laboratory of Food Safety and Quality, Faculty of Food Science and Technology, Universiti Putra Malaysia. Both isolates were isolated from milk (Lye *et al*., 2013). Bacterial strains were growth in Mueller-Hinton broth (MHB) medium (Difco Becton Dickinson, Sparks, MD) or MHB supplemented with 1.5% of bacterial agar (MHA) at 37°C for over night.

In vitro susceptibility test

Disc-diffusion method

B. rotunda extract was tested for antibacterial activity against three *E. coli* strains using disc-diffusion method. An overnight culture of each strain was spread on MHA using sterile cotton swab. Sterile paper disc (6 mm) was impregnated with 10 µL of 1% extract. A 1% of chlorhexidine was included as a positive control. The plates were incubated at 37°C for 24 hours and observed for any clear zone surrounding the paper disc (including the disc diameter). The test was perfomed in duplicate to verify the results.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) evaluation

The minimum inhibitory concentration (MIC) of *B. rotunda* extract against each strain was performed according to a method described in the guidelines of Clinical Laboratory Standard Institute M7-A6 (2003). Briefly, test was done in a 96-well round bottom microtiter plate using standard broth microdilution methods with an inoculum of approximately 10^6 cfu/mL. A two fold dilution of *B. rotunda* extract stock solution or chlorhexidine was mixed with the test organisms in MHB medium. Column 12 of the microtiter plate contained the highest concentration of extract or chlorhexidine, while column 3 contained the lowest concentration of extract or chlorhexidine. Column 2 served as positive control for all samples (only medium and inoculum or antimicrobial agent-free wells), and column 1 was the negative control (only medium, no inoculum, and no antimicrobial agent). Microtiter plates were incubated aerobically at 37°C for 24 h. The MIC was defined as the lowest concentration of antimicrobial agent that resulted in the completed inhibition of visible growth. The extract was diluted in 10% DMSO and the concentration of DMSO is decreased serially after two fold dilutions in the test wells.

Minimum bactericidal concentration (MBC) was defined as the lowest concentration of antimicrobial agent at which no growth occur in the MHA plate. MBC was determined by sub-culturing the suspension (10 µL) from each wells on MHA plate. All the wells include positive control (column 2) and negative control (column 1) were pipetted on the agar plate. The plates then incubated at 37°C for 24 hours or until growth seen at positive control. MBC was tested for all bacteria strains.

Time-kill curve

Time–kill assays were performed in MHB medium, according to the method of Lorian (2005) and Pankey and Ashcraft (2009), with modification. Briefly, the adjusted inoculum suspension of 5×10^7 cfu/mL was diluted 1: 10 in MHB medium to a final concentration of 5×10^6 cfu/mL. Each concentration of *B. rotunda* extract was diluted 1: 10 in MHB medium containing 5×10^6 cfu/mL. This yielded an initial inoculum of 4.5×10^6 cfu/mL. Final concentrations of *B. rotunda* extract were 0× MIC, 0.5× MIC, 1× MIC, and 2× MIC for each *E. coli* isolate. Cultures (5 mL final volume) were incubated at 37°C with 200 rpm agitation. At pre-determined time points (0 and 30 min as well as 1, 2, and 4 h), 100 µL aliquots were removed and transferred to centrifuge tubes, centrifuged (3900 rpm at 4°C for 1 min) and rinsed twice with 900 µL of sterile distilled water to obtain *B. rotunda* extract-free cells. Pellets were suspended in 10 mL of MHB medium and serially diluted. An appropriate volume (25, 50 or 100 µL, depending on the dilution and the concentration of *B. rotunda* extract) was spreaded onto MHA plates and incubated at 37°C for 48 h or more, until the colonies appeared on the plates, to

determine the number of cfu/mL. Assays were carried out on three different occasions, in triplicate.

Results and Discussion

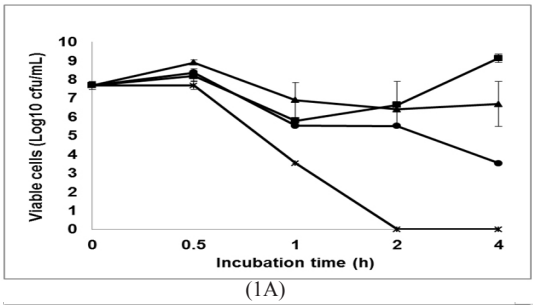
In the present study, methanol was chosen as the solvent for extraction of *B. rotunda*. It was because the methanolic extracts of plants usually have better antibacterial activity compared to other solvent such as hexane and water (Erlina *et al*., 2012). Furthermore, a study by Cowan (1999) concluded that the phytochemical compounds are more soluble in moderate polar organic solvent even though it is agreed that there are active and non-active compound present in the plant.

The antibacterial activities of the methanolic extract of *B. rotunda* with concentration of 10 mg/mL or 1% against *E. coli* strains are summarized in Table 1. The inhibition zone of *B. rotunda* extract against *E. coli* ATCC 25922, *E. coli* O157:H7-1 and *E. coli* O157:H7-2 was 9.0 mm, 7.0 mm, and 7.0 mm, respectively. The principle of disc-diffusion method is the larger the inhibition zone meaning has the greater antibacterial activity. Thus, *B. rotunda* extract was more effective against standard *E. coli* ATCC 25922 compared to *E. coli* O157:H7-1 and *E. coli* O157:H7-2 isolated from milk. Lye *et al.* (2013) reported that *E. coli* O157:H7-1 and *E. coli* O157:H7-2, are antibiotics-resistant bacteria. Roopnarin *et al*. (2005) and Hleba *et al.* (2010) also proved that *E. coli* isolated from milk products are resistant to ampicillin and chloramphenicol. It was supported by Alonso *et al.* (2000) and Sader *et al.* (2002) which *E. coli* has been reported to resist many antibiotics that are frequently used or available in the market. Shahedur *et al*. (2011) shows that no inhibition zone of plants extracts detected against resistant *E. coli*, except for *Citrus aurantifolia* that give 7-11 mm inhibition zone. Moreover, MIC and MBC of *B. rotunda* extract against standard *E. coli* ATCC 25922 were 0.019 mg/mL and 0.039 mg/mL, respectively. In contrast, MIC and MBC of *B. rotunda* extract against *E. coli* O157:H7-1 and *E. coli* O157:H7-2 was 2.5 mg/mL and 5.0 mg/mL, respectively. These result supported that *E. coli* O157:H7-1 and *E. coli* O157:H7-2 are antibiotics-resistant bacteria. However, *B. rotunda* extract showed stronger antibacterial activity against *E. coli* O157:H7-1 and *E. coli* O157:H7-2 compared with the clove extract which had the MIC of 10 mg/mL against *E. coli* O157:H7 (Kim *et al*., 2011).

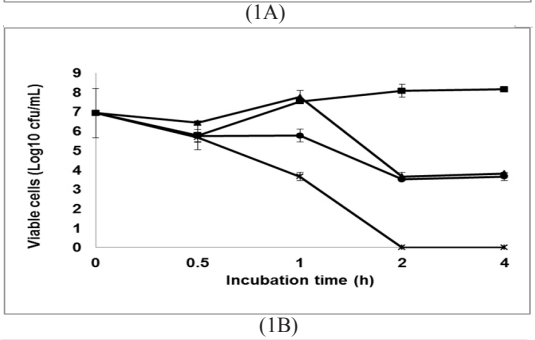
In the time–kill assays, *B. rotunda* extract significantly inhibited *E. coli* growth as compared to control cultures (Fig. 1). The bactericidal activity of *B. rotunda* extract was fast-acting against *E. coli* ATCC 25922, *E. coli* O157:H7-1 and *E. coli* O157:H7-2; the reduction in the cfu/mL was ≥3 log units (99.9%) at *B. rotunda* extract concentration of 1× MIC. The bactericidal endpoints for all *E. coli* tested were reached after 2 hours of incubation at *B. rotunda* concentration of 2× MIC. This study shows that *B. rotunda* extract has a potential antibacterial

Table 1. Inhibition zone, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Boesenbergia rotunda* extract against *Escherichia coli*

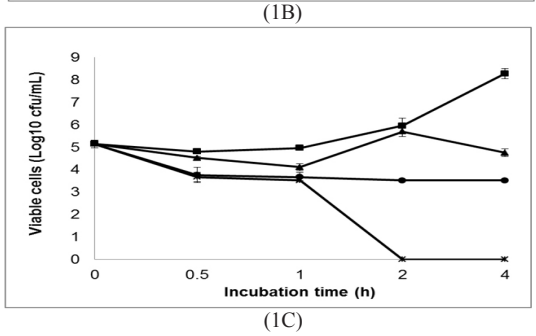
***Escherichia coli* strains**	**Inhibition zone (mm)**	**MIC (mg/mL)**	**MBC (mg/mL)**
Escherichia coli ATCC 25922	9.0	0.019	0.039
Escherichia coli O157:H7-1	7.0	2.5	5.0
Escherichia coli O157:H7-2	7.0	2.5	5.0



(1A)



(1B)



(1C)

Figure 1. Time – kill plots of *E. coli* ATCC 25922 (1A), *E. coli* O157:H7-1 (1B), and *E. coli* O157:H7-2 (1C), following exposure to *B. rotunda* extract at 0× MIC (filled squares, control), 0.5× MIC (filled triangles), 1× MIC (filled circles), and 2× MIC (open diamonds) after endpoint (4 h), *E. coli* ATCC 25922 (0, 0.009, 0.019, 0.038 mg/mL); *E. coli* O157:H7-1 (0, 1.25, 2.5, and 5 mg/mL); *E. coli* O157:H7-2 (0, 1.25, 2.5, and 5 m g/mL). Values given in the brackets after species are 0× MIC (control), 0.5× MIC, 1× MIC, and 2× MIC, respectively.

activity against *E. coli* strains.

Conclusion

B. rotunda extract exhibits its potential as antimicrobial activity against a species of important foodborne pathogen, *E. coli.* MIC and MBC values of *B. rotunda* extract range 0.019 mg/mL – 2.5 mg/mL and 0.039 mg/mL – 5.0 mg/mL, respectively. *B. rotunda* extract can kill the *E. coli* strains after 2 hours at a concentration of 2× MIC. Further study of this extract on other foodborne pathogens such as *Staphylococcus aureus*, *Salmonella* Typhimurium, *Bacillus cereus*, and *Vibrio parahaemolyticus* would be more interesting.

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