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Antifungal activity of *Boesenbergia rotunda* (temukunci) extract against filamentous spoilage fungi from vegetables

[1]Zakuan, Z., [1]Mustapa, S. A., [1,2]Sukor, R. and [1,3*]Rukayadi, Y.

[1]Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
[2]Laboratory of Food Safety and Food Integrity, Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia,
[3]Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia.

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Abstract

The filamentous spoilage fungi in vegetables can lead to significant impact in food and economic loss. In order to overcome this problem, chemical fungicide has been implemented in vegetable farming and processing but it causes problems towards environment and food safety. Thus, the utilization of natural products such as plants extracts, which exhibit antimicrobial and antifungal activity, is more acceptable to solve this problem. The aim of this study is to investigate the antifungal activity of *Boesenbergia rotunda* extract against ten filamentous spoilage fungi isolated from five vegetables. The extract was used to treat fungal isolates from vegetables; CRb 002 (*Penicillium* sp.), CHa 009 (*Aspergillus* sp.), TMa 001 (*Geotrichum* sp.), TMa 002 (*Aspergillus* sp), ONb 001 (*Aspergillus* sp.), WBb 003 and WBb 004 (*Fusarium* sp.) WBb 007 (unidentified), WBb 008 (*Aureobasidium* sp.) and WBb 010 (*Penicillium* sp.). The results showed that the yield of the extract of *B. rotunda* using ethanol (95%) was 11.42% (w/v). The 10% of *B. rotunda* extract exhibited antifungal activities against ten filamentous fungi after 5 days treatment with growth reduction of 41.56%, 30.68%, 86.20%, 50.62%, 26.67%, 47.44%, 50.74%, 36.39%, 42.86%, and 39.39% for WBb 008, WBb 004, WBb 007, WBb 003, CRb 002, WBb 010, CHa 009, TMa 001, ONb 001, and TMa 002, respectively. *B. rotunda* extract showed highest antifungal activity against fungi isolated from winged bean (WBb 007) with percentage reduction in growth was 86.20%, while the lowest activity was against fungi isolated from the carrot (CRb 002) with 26.67% reduction in growth. Generally, the TPC of fungi in the vegetable samples were reduced after treatment with 5% of *B. rotunda* extract at 5 min and 10 min of exposure time. The results suggested that *B. rotunda* extract has high potential to become natural food preservative which can reduce the fungi spoilage of vegetables.

Introduction

Microbial spoilage in the vegetables becomes a severe problem especially for post-harvest crops (Tournas, 2005). These losses have been estimated up to 40% in developing country especially at post-harvest and processing stage (FAO, 2011). In another study, almost 20% of crops worldwide have been attacked by fungal infections and these problems were occurred due to low pH, moisture content and high nutrient in the vegetables (Pitt and Hocking, 2009). *Alternaria, Aspergillus, Cladosporium, Colletotrichum, Phomopsis, Fusarium, Penicillium, Phoma, Phytophthora, Pythium* and *Rhizopus* spp., *Botrytis cinerea, Ceratocystis fimbriata, Rhizoctonia solani* and *Sclerotinia sclerotiorum* are the common fungi that infect vegetables (Tournas, 2005).

Chemical fungicide is one of the effective techniques to overcome this problem. Furthermore, it becomes primary method due to low cost and easy to use (Xia *et al*., 2006). However, it contains hazardous substances which can lead to environmental problems. In addition, it may contaminate the food chain when fungicide residues remain in the food being consumed by human (European Food Safety Authority, 2011). Other than that, ozone treatment also can be applied to reduce spoilage fungi in the vegetables (Yadav, 2009; Gabler *et al.*, 2010). In addition, this method was more tolerable by the producers due to the free of chemical residues (Frank *et al*., 2010). Nevertheless, ozone treatment method may cause harm to human when used at high concentration and exposure. Therefore, alternative methods which are less harmful to human, plants and

*Corresponding author.
Email: *yaya_rukayadi@upm.edu.my*

an environment are needed.

Natural plant extract is an alternative method to replace the use of chemical and ozone treatment in treating spoilage fungi in vegetables. It is because plants have a variety of secondary metabolites, such as tannins, terpenoids, alkaloids, flavonoids and glycosides which display antimicrobial properties. (Valentin *et al*., 2010; Radulovic *et al*., 2013). Meanwhile, Ruszkowska and Wrobel (2003) also reported that compounds such as 2-decanone, hydroxydihydrocornin-aglycones, indole derivatives and flavanone isolated from plants showed antifungal activity. Other than that, essential oil from plant extraction process also had an antifungal activity that useful to overcome the problems (Combrink *et al*., 2011; Prakash *et al*., 2015).

Boesenbergia rotunda consists of essential oil and many flavonoids that can be used as anti-inflammatory, antibacterial and antifungal, antioxidant and many more (Chahyadi *et al*., 2014). Furthermore, to best of our knowledge the antifungal activity of *B. rotunda* extract against the filamentous fungi isolated from vegetables was not widely studied. Thus, antifungal activities of *B. rotunda* extract against filamentous fungal spoilage isolated from vegetables have been investigated using different concentration with different exposure times. Therefore, this study is crucial to examine the ability of *B. rotunda* extract to inhibit fungal growth.

Materials and Methods

Plant sample

Dried samples of rhizomes of *Boesenbergia rotunda* were purchased from Herbal Market, Pasar Baru, Bandung, Indonesia. The specimen was deposited at Laboratory of Natural Product, Institute of Bioscience (IBS), Universiti Putra Malaysia.

Preparation of Boesenbergia rotunda *extract*

The extraction of *B.rotunda* was done according to Rukayadi *et al.* (2008) with some modification. Fifty gram of dried rhizome of *B. rotunda* was pulverized into fine powder. Then, the powdered rhizome was extracted using 200 ml of 95% ethanol for 4 days at room temperature with occasional agitation. The extract was filtered by using Whatman filter paper No. 1 followed by concentration by rotary vacuum evaporator (Heidolph VV2011, Schwabach, Germany) at 40°C. The extraction process was repeated three times. The concentrated extract was dissolved in 100% dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) to yield a concentration of 100 mg/ml or 10% (w/v). It was then further diluted to obtain 10 mg/ml or 1% (w/v) of *B. rotunda* extract in 10% (v/v) DMSO. 10% DMSO was not found to kill filamentous fungi tested (Rukayadi *et al*., 2013).

Table 1. Fungi isolates and its genera

Source of isolation	Isolates code	Genera
Carrot	CRb 002	*Penicillium* sp.
Chili	CHa 009	*Aspergillus* sp.
Tomato	TMa 001	*Geotrichum* sp.
	TMa 002	*Aspergillus* sp.
Onion	ONb 001	*Aspergillus* sp.
Winged bean	WBb 003	*Fusarium* sp.
	WBb 004	*Fusarium* sp.
	WBb 007	Unidentified
	WBb 008	*Aureobasidium* sp.
	WBB 010	*Penicillium* sp.

Inoculum preparation

Ten filamentous fungi (Table 1) were previously isolated from five types of vegetables, i.e: carrot, chilli, onion, tomato and winged bean, and deposited at Microbiology Laboratory, Institute of Bioscience (IBS), Universiti Putra Malaysia. The fungi were cultured and maintained on potato dextrose agar (PDA) (Difco, sparks, MD) aerobically for 7 days. The cultures were stored at 4°C until further usage.

Antifungal activity by Boesenbergia rotunda

The agar well diffusion method (Wanchaitanawong *et al*., 2005) was modified to test antifungal activity of *B. rotunda* extracts. Four fungal disc (6 mm) were cut at the periphery of a seven-day-old culture using sterile cork borer. A fresh PDA plates were also punctured with the similar manner. Then, the fungi disc were transferred into each well of PDA plates. Consequently, 50 µl of 10% (w/v) extract, 1% (w/v) extract, 10% (v/v) dimethyl sulfoxide (DMSO) and 20 µl of 1% (v/v) Amphotericin B (Amp B) were pipetted onto each of the well. Ten percent DMSO and 1% Amphotericin B were used as negative and positive control respectively. The agar plates were incubated at 30°C for 4 days. All test were repeated three times with three replications each (n= 3 × 3). The diameter of fungi colonies was measured and percentage of reduction fungi growth was calculated by using a formula below.

$$I\,(\%) = [(C - T) / C] \times 100$$, where:

T = test
C= control
I = percentage reduction of fungi growth
(Pandey *et al*., 1982; Luo *et al.*, 2005)

Table 2. Diameter of fungi colonies after treatment with 10% DMSO, 1% Amp B, 1% of *B. rotunda* extract and 10% of *B. rotunda* extract

Fungi or types of vegetables	10% DMSO	1% Amp B	1% extract	10% extract
Carrot (CRb 002)	1.50±0.20^{a}	1.20±0.07^{a}	1.45±0.00^{a}	1.10±0.30^{a}
Chilli (Cha 009)	2.03±0.04^{a}	1.33±0.30^{ab}	1.70±0.40^{ab}	1.00±0.00^{b}
Onion (ONb 001)	2.10±0.00^{a}	1.38±0.20^{b}	1.70±0.20^{ab}	1.20±0.07^{b}
Tomato (TMa 001)	3.38±0.40^{a}	3.30±0.10^{a}	3.20±0.00^{a}	2.15±0.20^{b}
Tomato (TMa 002)	1.65±0.10^{a}	1.13±0.04^{bc}	1.45±0.07^{ab}	1.00±0.07^{c}
Winged bean (WBb 003)	4.05±0.30^{a}	3.65±0.30^{a}	3.53±0.40^{a}	2.00±0.50^{b}
Winged bean (WBb 004)	4.40±0.90^{a}	3.80±1.40^{a}	4.23±0.20^{a}	3.05±0.00^{a}
Winged bean (WBb 007)	4.35±0.00^{a}	3.33±0.03^{b}	3.0±0.2^{b}	0.60±0.00^{c}
Winged bean (WBb 008)	3.85±0.40^{a}	3.30±0.10^{ab}	2.55±0.10^{bc}	2.25±0.10^{c}
Winged bean (WBb 010)	2.15±0.07^{a}	1.43±0.10^{b}	1.63±0.20^{ab}	1.13±0.20^{b}

Means that do not share the same superscript letter are significantly different ($P < 0.05$)

Effect of Boesenbergia rotunda *extract on the number of spoilage fungi of vegetables*

The treatment of selected vegetables, i.e., carrot, chilli, onion, tomato and winged bean was done to evaluate the effects of these extracts on natural filamentous spoilage fungi present on the surface of vegetables. The effects were evaluated in the means of total plate count (TPC) of fungi. A total of 5 g of the each of the listed vegetable samples were cut using sterile knife separately to prevent cross contamination. Then, each of the sample were treated with 45 ml of tap water , 0% (only sterile distilled water), 0.05% (w/v), 0.5% (w/v) and 5% (w/v) in 10% DMSO of *B. rotunda* extract at different time exposure which were 5 min and 10 min After that, the samples were dried on sterile filter paper before homogenized using a stomacher at 250 rpm for 2 min. An aliquot of 20 µl from serial dilutions of each treatment was plated onto PDA and incubated at 30 ˚C for 5 days. The number of colonies were counted and reported as Log CFU/ml. The tap water and sterile distilled water were used as positive and negative control respectively. Each of the assays were carried out twice with duplicates (n = 2× 2).

Statistical analysis

The analysis of variance (ANOVA) was applied to determine their significance differences for means for each treatment. The statistical analysis was used the Tukey's test (MINITAB) to determine the level significant difference between each treatment. Then, the results were interpreted as means ± standard deviation (SD) of duplicate analysis. The results for each treatment in application assay were calculated and interpreted in Log CFU/ml value.

Results and Discussion

The sample of *Bosenbergia rotunda* was extracted in three series of extraction. The weight of dried sample that was used for extraction was about 50.00 g. After the extraction process through the rotary vacuum evaporator the total weight of yield extracted from *B. rotunda* was 5.71 g (11.42% of yield). From previous studies, 12% and 9.49% yield of *B. rotunda* extract were obtained using ethanol as solvent in solvent extraction evaporation and distillation methods, respectively (Salama *et al*., 2013; Woo *et al.*, 2015). Thus, differences of yield for each study were obtained because differences in volume of solvent and method used. The extraction was repeated three times due to significant amount of active compounds which may be left behind in the first soaking process (Azmir *et al.*, 2013). Ethanol was selected as the solvent for extraction process due to its ability to extract flavonoid compounds

Table 3. Percentage reduction of fungi growth

Fungi	Percentage reduction of fungi growth for 10% extract (%)
CRb 002	26.67[g]
Cha 009	50.74[b]
ONb 001	42.86[d]
TMa 001	36.39[e]
TMa 002	39.39[e]
WBb 003	50.62[b]
WBb 004	30.68[f]
WBb 007	86.20[a]
WBb 008	41.56[d]
WBb 010	47.44[c]

Means that do not share the same superscript letter are significantly different (P < 0.05)

that are mainly responsible for *B. rotunda* biological activities (Mahmood *et al.*, 2010). In another study, essential oil of *B. rotunda* using 95% ethanol as solvent showed potent activity against *Candida albicans* (Taweechaisupapong *et al*., 2010).

The concentration of 1% and 10% of *B. rotunda* extract were applied against ten filamentous fungi spoilage isolated from carrot, chilli, onion, tomato, and winged bean with 10% DMSO as negative control and 1% Amp B as positive control. The effect of all the treatments on fungi growth was evaluated by measuring the diameter of fungi colonies for each treatment. The results for diameter of fungi growth for each treatments (10% DMSO, 1% AmpB, 1% extract and 10% extract) against each fungi and the percentage of reduction of fungi growth for 10% of *B. rotunda* extract were depicted in Table 2 and Table 3, respectively. Based on the results, 10% extract of *B. rotunda* showed strongest antifungal activity against filamentous fungi isolated from winged bean (WBb 007) which is 86.20% while the lowest was against fungi isolated from the carrot (CRb 002) with 26.67% reduction in growth. The 10% extract of B. rotunda reduced significantly (P < 0.05) all the filamentous fungi growth from tomato (TMa 001 and TMa 002), chilli (CHa 009), winged bean (WBb 003, WBb 007,WBb 008, and WBb 010) and onion (ONb 001) as compared to DMSO (negative control) in the experiment. This is in agreement with the previous studies by Pattaratanawadee *et al*. (2006) who reported that 10%, 8% and lesser of ethanolic extract of B. rotunda had inhibitory efficiency against several filamentous fungi, such as *Aspergillus parasiticus, Aspergillus niger* and *Fusarium oxysporum,* respectively. In another study, Jantan *et al*. (2003) mentioned that the oil of *B. rotunda* rhizomes was effective against dermatophytes, filamentous fungi and yeast including *C. albicans* and *C. neoformans.* This spice extract has the ability to alter the morphology of the hyphae by collapsing the rigid structure (Rasooli *et al*., 2005).

Application of *B. rotunda* extract on the vegetables were done at different series of concentration which are tap water, 0%, 0.05%, 0.5%, and 5% at different exposure time (5 min and 10 min) to evaluate the effect of extract on TPC of fungi. The results for each of treatments were calculated and interpreted in Log CFU/ml value. Based on the result (Table 4), the number of total plate count (TPC) from winged bean showed a reduction using tap water and different concentrations of the extract at 10 min exposure time. The number of TPC (Log CFU/ml) for winged bean sample after treatment with tap water, 0.00%, 0.05%, 0.50% and 5% extracts were 6.18 ± 0.00, 5.71 ± 0.12, 5.70 ± 0.01, 5.62 ± 0.01, and 5.43 ± 0.20, respectively. The number of total plate count (Log CFU/ml) for winged bean sample was reduced significantly ($p < 0.05$) from 6.18 ± 0.00 using tap water to 5.43±0.20 using 5% extract at 10 minutes exposure time. Similar results were obtained to chilli samples, where the number of TPC was reduced significantly ($p<0.05$) at 5 min and 10 min exposure time from 4.90±0.12 and 4.67±0.20 sing tap water to 4.00±0.00 and 3.85±0.20 using 5% extract, respectively. Generally, the number of total plate count from vegetables can be reduced using 5% extract.

Referring to the previous studies, boesenbergia, cardamonin, pinostrobin, pinocembrin, panduratin A and 4-hydroxypanduratin A are active compounds that cause this plant to possess antifungal activity, anti-dengue N2SB/NS3 protease, antibacterial, anti-inflammatory, anticancer and antioxidant activity (Eng *et al*., 2012). Meanwhile, Pompimon *et al*. (2009) and Seniya *et al.* (2013) had stated that antifungal activity of *B. rotunda* were due to the existence of 4-hydroxypanduratin, pinostrobin and pinocembrin. Phenolic compounds such as flavonoid in the *B. rotunda* also have been associated to in-vitro antimicrobial properties. However, the mechanism of secondary metabolites to act as antimicrobial agents is unclear but various potential modes of action have been proposed. Firstly, the secondary metabolites cross the cell membranes into the interior of the cell and lead to the critical intercellular functions (Cristani *et al*., 2007). Secondly, cell death due to

Table 4. Total plate count of fungi (TPC) for each vegetable in each treatment at 5 min and 10 min (Log CFU/ml)

Samples	Exposure time	Tap water	0.00%	0.05%	0.50%	5.00%
Carrot	5 min	4.94±0.09^{a}	5.00±0.12^{a}	5.36±0.30^{a}	4.87±0.13^{a}	5.23±0.16^{a}
	10 min	5.22±0.06^{a}	4.94±0.28^{a}	5.36±0.13^{a}	5.40±0.11^{a}	5.62±0.23^{a}
Chilli	5 min	4.90±0.12^{b}	4.70±0.00^{b}	5.49±0.04^{a}	5.44±0.23^{a}	4.00±0.00^{c}
	10 min	4.67±0.20^{bc}	4.57±0.04^{c}	5.25±0.12^{a}	5.13±0.04^{ab}	3.85±0.20^{d}
Tomato	5 min	4.00±0.00^{ab}	4.48±0.00^{a}	3.70±0.00^{b}	4.15±0.21^{ab}	3.85±0.21^{b}
	10min	3.70±0.00^{a}	4.00±0.00^{a}	3.85±0.21^{a}	3.85±0.21^{a}	3.85±0.21^{a}
Onion	5 min	5.38±0.01^{a}	4.83±0.25^{a}	5.00±0.06^{a}	5.23±0.14^{a}	5.18±0.22^{a}
	10 min	5.02±0.06^{ab}	4.90±0.04^{b}	5.44±0.00^{a}	5.25±0.24^{ab}	5.41±0.04^{ab}
Winged Bean	5 min	6.22±0.01^{a}	5.78±0.10^{ab}	5.77±0.03^{ab}	5.78±0.27^{ab}	5.58±0.13^{ab}
	10 min	6.18±0.00^{b}	5.71±0.12^{b}	5.70±0.01^{b}	5.62±0.01^{b}	5.43±0.20^{a}

Mean values ± standard deviation with different superscript letters in the same row are significant different ($P < 0.05$).

the structural damage on cell membranes caused by these compounds or these compounds act on the hyphal cell wall of fungi and lead to the collapse of the fungal mycelium (Gill and Holley, 2006; Sharma and Tripathi, 2006).

All of the results obtained showed that *B. rotunda* extract can be used to inhibit filamentous fungi isolated from vegetables and reduce the number of total fungal colony from the vegetables. Based on the application of vegetables in the extracts, 5% extract can be used to reduce the number of spoilage fungi for several vegetables within a short time (5 min) but it might be costly to commercialize it. Thus, 0.5% extract was recommended to use as vegetable detergent to reduce the number of spoilage fungi for several vegetables for 10 min.

Conclusion

In conclusion, *Bosenbergia rotunda* extract has high potential to be developed as natural antifungal to reduce spoilage of vegetables caused by fungi as its antifungal activity was demonstrated. However, in depth study regarding this extract such as stability of the extract and its constituents must be done in order to develop high commercial value of natural food preservatives.

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