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Existence of bioactive flavonoids in rhizomes and plant cell cultures of *Boesenbergia rotunda* (L.) Mansf. Kulturpfl.

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Abstract

Callus and cell suspension cultures were explored as alternative sources of selected flavonoids and compared to those produced from rhizome of *Boesenbergia rotunda* (L.) Mansf. Kulturpfl. *B. rotunda* or fingerroot ginger is a medicinally important Zingiberaceae species. HPLC analysis showed the presence of alpinetin, pinocembrin, cardamonin, pinostrobin and panduratin A in the rhizomes, callus and cell suspension cultures of *B. rotunda*. Among the flavonoids, pinostrobin was predominant compared to panduratin A from the three sources. The rhizome extract of *B. rotunda* produced the highest quantity: a total of 5 selected flavonoids at 12975.52 ± 71.78 µg g^{-1} dry weight (DW). In contrast, callus and cells suspension extracts yielded 120.61 ± 0.01 and 3.14 ± 0.12 µg g^{-1} DW of the total selected flavonoids, respectively. However, this is the first report on the production of alpinetin, pinocembrin, cardamonin, pinostrobin and panduratin A from *in vitro* cultures of *B. rotunda* (L.) Mansf. Kulturpfl. Although the amount of flavonoids from *invitro* cultures was not comparable to the rhizome, it is crucial to initially establish *invitro* cultures and to proof the existence of these compounds in the cultures. This is a pre-requisite to enhance the accumulation of targeted bioactive compound through either metabolic engineering or chemical elicitation in future studies.

Keywords: Medicinal plant; Plant cell cultures; Secondary metabolites; Zingiberaceae.
Abbreviations: DW - dry weight; HPLC - high performance liquid chromatography; MS - Murashige and Skoog; NAA - α-naphthalene acetic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid.

Introduction

Boesenbergia rotunda (L.) Mansf. Kulturpfl., a member of *Zingiberaceae* is an important medicinal plant. Traditionally, *B. rotunda* is used to treat diseases such as leucorrhoea, dysentery and inflammation (Burkill, 1935). The pharmacological significance of this plant is mainly due to the presence of flavonoid, flavones, essential oil and chalcones (Jaipetch et al., 1982, Trakoontivakorn et al., 2001). Fahey and Stephenson (2002) reported that pinostrobin (flavonone) has various activities including elevating the activity of an antioxidant enzyme, antispasmodic agent, reduce estrogen-induced cell proliferation, decreasing spontaneous contractions of intestinal smooth muscle and also mediating anti-inflammatory (Meckes et al., 1998, Le Bail et al., 2000, Wu et al., 2002). Cardamonin (flavonoid) displayed anti-HIV-1 protease inhibition (Tewtrakul et al., 2003) and was also found to be analgelsic and antipyretic (Pathong et al., 1989). Panduratin A (flavonoid) was found to reduce the development of human breast cancer and colon adenocarcinoma cell (Kirana et al., 2007), inhibit dengue-2 virus protease activity (Kiat et al., 2006), anti-aging activity (Shim et al., 2009) and have potential antibacterial and antiviral activities (Rukayadi et al., 2010, Wu et al., 2011). Owing to the various functional pharmaceutical activities, it is of great interest to develop suspension cultures for alternative production of secondary metabolites. Cell suspension cultures enable climate and plant source independent production of compounds. This cell suspension culture system is controlled and highly applicable in the development of large scale plant metabolite production. A well-established cell suspension system also allows for rapid enhancement of the production of desired compounds through metabolic engineering and chemical elicitation. The aim of this report is to profile the existence of alpinetin, pinocembrin, cardamonin, pinostrobin and panduratin A, in rhizomes, callus and cell suspension cultures of *B. rotunda*. This provides baseline information on the presence of the compounds produced in *in vitro* cultures which is crucial for further manipulation of the metabolic pathway in other relevant studies.

Results and Discussion

Calli of *B. rotunda* were induced from shoot base explants on MS medium supplemented with 1 mg L^{-1} NAA, biotin and IAA, 2 mg L^{-1} 2,4-D and 30 g L^{-1} sucrose. The percentage of explants that produced callus was 53.4%. Swelling of the explants was observed within one to two weeks, followed by emergence of callus after another two weeks. Callusing commenced at the cut surfaces of the explants and finally covered up the surface of the explants completely. After two to three times of subcultures, rapid increase in the callus mass was observed in the medium. The callus developed in this medium was mixed friable and compact, cream coloured

Table 1. Flavonoids levels in rhizome and in *in vitro* cultures

Compounds*	Amount of flavonoids from different sources		
	Rhizomes ($\mu g\ g^{-1}$ DW)	Callus ($\mu g\ g^{-1}$ DW)	Cells suspension ($\mu g\ g^{-1}$ DW)
Alpinetin	1139.24 ± 6.27^b	4.50 ± 0.00^a	0.30 ± 0.04^a
Pinocembrin	2613.77 ± 14.24^b	17.76 ± 0.09^a	1.02 ± 0.08^a
Cardamonin	57.20 ± 0.30^c	1.63 ± 0.00^b	0.24 ± 0.01^a
Pinostrobin	8220.72 ± 45.66^c	96.30 ± 0.00^b	3.15 ± 0.34^a
Panduratin A	944.58 ± 5.31^b	0.42 ± 0.00^a	0.20 ± 0.10^a
Total Flavonoids	12975.52 ± 71.78^c	120.61 ± 0.01^b	4.91 ± 0.57^a

*At p=0.05, significance differences were observed for each flavonoid contents from different sources.

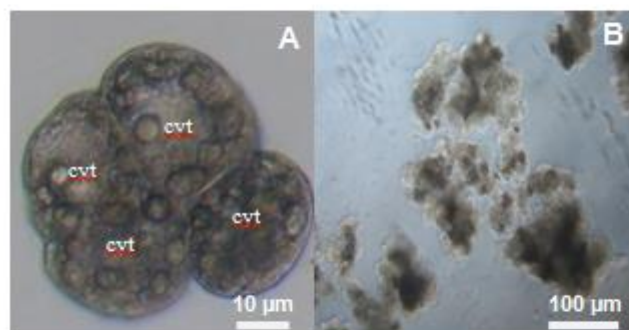

Fig 1. Multiplication and proliferation of dense, cytoplasmic cells of the suspension cultures. (*A*) proliferating cell suspension cultures after sieving using 450 µm stainless steel filter under inverted microscope (*B*) cell suspension at day 14th of culture showing aggregated cells without debris. cyt; cytoplasm.

callus, which was sub cultured at four weeks intervals. At this stage, the callus was suitable for further subculture or establishment of suspension cultures. The callus growth rate was found to be 0.38 ± 0.004 g per week. The growth rates were comparable for the second and third subcultures which subsequently decreased gradually (data not shown). Malamug et al., (1991) and Tan et al., (2005) showed a similar pattern of growth proliferation for *Zingiber officinale* and *B. rotunda*. Sigmoidal growth pattern showed that the cell suspension is a reproducible and sustainable system. Cell suspension cultures were light yellow in colour and contained homogenous fine cells within three months of initiation (Fig 1A and B) which was ideal for suspension culture systems. Through microscopic observation, the cells in suspension cultures showed vigorous proliferation. On the 14th day after subculture, cell suspensions comprised of a large portion of cytoplasmic rich, spherical cells (Fig 1A and B). This finding was comparable with previous reports (Côte et al., 1996; Jalil et al., 2008). Plant cell suspension cultures usually have an aggregating tendency to form cohesive cell aggregates or larger callus clusters during culture because some types of polysaccharides excreted by plant cells increase the viscosity of the culture system at later stages of the culture (Zhao et al., 2001). Cytoplasmic cells indicated vigorous growth due to the active cytoplasmic streaming present in the cells. The chemistry of *B. rotunda* metabolites has been widely explored (Trakoontivakorn et al., 2001, Kiat et al., 2006, Chee et al., 2010). Despite numerous reports on the phytochemistry and biological properties of *B. rotunda*, only one in house communication was found on the production of bioactive compounds using *B. rotunda in vitro* cultures (Khalid et al., 2010). Productions of compounds in field grown rhizomes are subjected to environmental factors, abiotic and biotic stress, geographical locations which will influence quality and quantity compound of interest. The amounts of flavonoids in each sample are presented in Table 1. Fig 2 shows HPLC chromatogram of the samples. The main compound in all of the samples was pinostrobin. It was observed that the *in vitro* cultures of *B. rotunda* produced similar compounds as in intact rhizomes although the quantity differed. Among the *in vitro* cultures, callus was found to produce the highest amount of flavonoids. Callus cultures are usually more productive than cell suspensions of the same origin, cultivated on the same medium composition. The low secondary metabolite production in *B. rotunda* cell suspension cultures could be due to undifferentiated stage used for the extraction. Synthesis of secondary metabolites has been linked to the degree of differentiation of individual cells which lacked either single specialized cells, cell compartments and tissues or a specialized part of organs that serve as the synthesis and storing sites for secondary metabolites (Endress, 1994). Other than differentiation stages, static media can accumulate higher flavonoids than in liquid medium. Possible secretion of flavonoids into the surrounding medium as well as the degradation of the product could be the contributing factors for low flavonoids yield in cell suspension cultures. This has been shown in *Alpinia officinalis* culture where 29% of oleanolic acid glycosides were transported out of the leaf protoplasts through the cell membrane (Omar, 2003). Secretion of terpenoids such as essential oil was reported by Evert (2006). In contrast to the present study, alkaloids such as corybulbine, corydaline and cavidine produced in intact tubers, were absent in the callus of *Corydalisambigua* (Hiraoka et al., 2004), while azadirachtin was higher in the callus cultures than the differentiated tissues of *Azadirachta indica* (Wewetzer, 1998). The production of antihypertensive alkaloid was equal in the callus and leaf tissue of *Catharanthus roseus* (Namdeo et al., 2006). The unexpected accumulation of secondary metabolite in the *in vitro* tissue culture system may be due to many factors such as plant growth regulators, nutrient medium, carbohydrate source employed and cell line.

Even though callus cultures were superior to suspension cultures in terms of flavonoids production, cell suspension culture systems enable reproducible production of calli in abundance at a rapid phase.

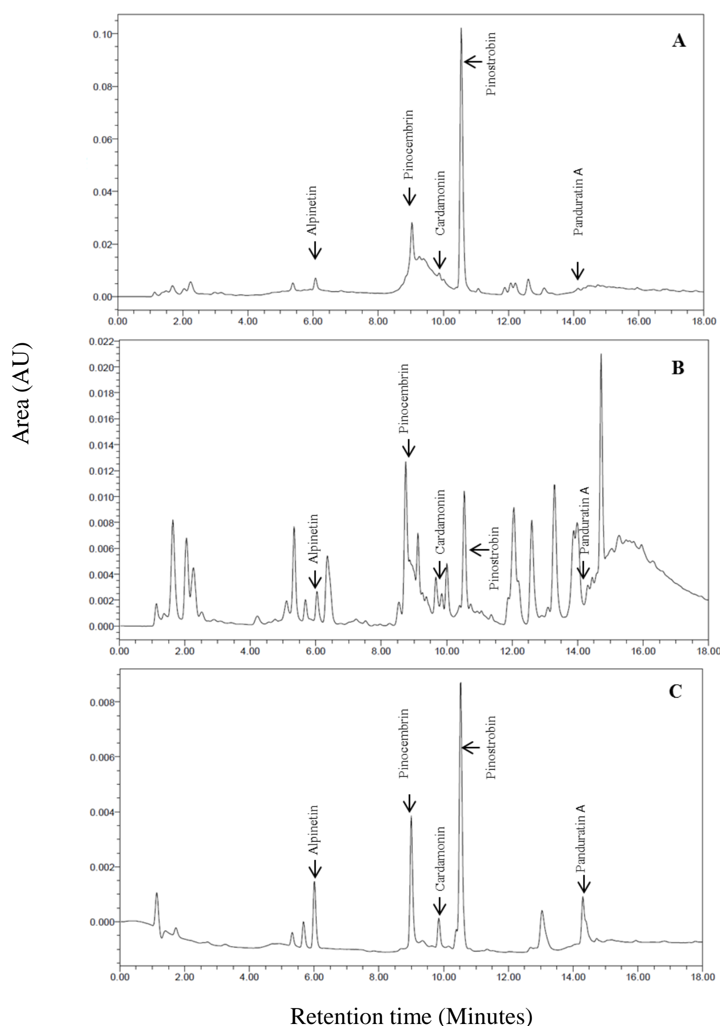

Fig 2. HPLC chromatograms of *B. rotunda* showing retention times of alpinetin, pinocembrin, cardamonin, pinostrobin and panduratin A at 6.10, 9.07, 9.88, 10.58 and 14.33 min, respectively. AU - absorbance units. (*A*), callus (*B*), cell suspension (*C*), rhizomes.

It is crucial to increase and accumulate maximum active biomass producing metabolite by cell suspension cultures in order to achieve the highest possible production of bioactive compounds from undifferentiated cells where it is only feasible with critical cell biomass. The balance between rapid cell growth and high compound production is crucial. Additionally, cell suspension cultures system is preferable for up scaling purposes where it allows reproducibility of consistent and constant source of desired compounds.

Material and methods

Plant materials

Three different sources of plant materials were used in this study i.e. rhizomes, callus and cell suspension cultures. Field grown rhizomes were washed thoroughly under running tap water for 30 min before being sliced and oven dried. The establishment of callus culture was carried out according to the procedures outlined in Yusuf et al. (2011). All experiments were performed with 30 replicates. One to 1.5 cm in diameter sized calli were cultured in initiation media and subcultured every four weeks on propagation medium (Tan et al, 2005). The callus growth rate was calculated using the equation described by Yokata (1999). Two g of calli (fresh weight, FW) were inoculated into 50 cm^3 Murashige and Skoog (1962), (MS) liquid media supplemented with 1 mg L^{-1} NAA and biotin, 2 mg L^{-1} 2,4-D, 0.1 mg L^{-1} L-glutamine and myo-inositol and 30 g dm^{-3} sucrose. The cultures were incubated in 16/8 h light and dark conditions with continuous shaking (80 rpm) and were maintained through routine transfer of small aliquots of the suspension into a fresh medium at every two weeks. Independent experiments were carried out in a minimum of three replicates.

Extraction

For the extraction of five selected flavonoids, rhizomes, callus and cells from suspension cultures were oven dried at 38 °C and pulverized. Powdered samples (1.0 g) were soaked in 100 ml of methanol for 72 h and filtered through a Whatman No. 1 filter paper. The filtrates were concentrated using a rotary evaporator (BÜCHI Rotavapor R-114). The slurry residue was then partitioned against an equal volume of ethyl acetate and water. Ethyl acetate fraction was again evaporated. The mass of the partitioned ethyl acetate extract was recorded and re-dissolved in methanol at a ratio of 1.0 mg of extract to 0.2 ml methanol. This methanolic solution of the extract was filtered through 0.45 µm PTFE filter (Sartorius 13 CR) prior to HPLC injection.

HPLC analysis

Five flavonoids namely alpinetin, pinocembrin, cardamonin, pinostrobin and panduratin A were purified and crystallized using a modified method following Kiat et al., (2006). These flavonoids crystals were used as standards in the HPLC analysis. HPLC grade organic solvents i.e. ethyl-acetate, methanol and acetonitrile (Merck) and phosphoric acid (BDH) were used. Quantitative analyses of the five selected flavonoids were performed by a HPLC system (Waters, USA) equipped with W600E multisolvent delivery system, W2489 UV/visible detector, W2707 auto sampler and in-line de-gasser. The reverse column used was a Chromolith RP-18e, (100 x 4.6 mm i.d.; 2 µm) (Merck) provided with guard cartridges RP-18e (5 x 4.6 mm i.d.; Merck). The gradient solvents systems used for elution were a mixture of 0.1% phosphoric acid (A) and acetonitrile (B). Analyses of flavonoids were carried out by the following gradient elution (0.5 min, 80% A, 20% B; 4.5 min, 65% A, 35% B; 5.0 min, 40% A, 60% B; 8.0 min, 100% B), with a total run time of 18 min at a flow rate of 1.5 ml/min. Peak detection was performed at both 285 and 330 nm. The 0.02 ml samples were injected and identification of flavonoids was done by comparing the retention time of absorption spectra with those of reference standards. Quantification of alpinetin, pinocembrin, cardamonin, pinostrobin and panduratin A in the extracts was done using a standard calibration method applicable within the range of 0.002 to 1.0 µg. The data were acquired and processed by Empower 2® software and expressed as µg of each compound per gram dry weight (DW).

Data Analysis

Experimental data were subjected to the analysis of variance (ANOVA) and significant differences between mean values

were tested with the Tukey's Multiple Range Test at 95% confidence interval.

Conclusion

HPLC analysis displayed the presence of selected flavonoids in the callus and cell suspension cultures. Thus, it can be concluded that *in vitro* cultures system could produce high amount of metabolically active calli within a short period. Therefore it is essential to study the enhancement of metabolites production in *in vitro* systems particularly in cell suspension cultures in order to accomplish the maximum production of selected flavonoids.

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