



Journal of
**Pharmacology and
Toxicology**

ISSN 1816-496X



Academic
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Effects of Selected *Boesenbergia* Species on the Proliferation of Several Cancer Cell Lines

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ABSTRACT

The aim of this research was to determine the potential of *Boesenbergia* species collected from Sabah rainforest as anticancer remedy. This research was done to *Boesenbergia rotunda*, *B. pulchella*, *B. pulchella* var *attenuata* and *B. armeniaca* crude extracts on the *in vitro* proliferation of hormone-dependent breast cancer (MCF-7), non-hormone dependent breast cancer (MDA-MB-231), ovarian cancer (CaOV₃), colon cancer (HT-29) and cervix cancer (Hela) cell lines. The effects of the four *Boesenbergia* species (methanol extracts of leaves, stem and rhizome part) on the proliferation of MCF-7, MDA-MB-231, CaOV₃, HT-29 and Hela cell lines were measured using (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) MTT assay. The crude extracts with IC₅₀ value less than 100 µg mL⁻¹ were subjected to cell cycle analysis by using flow cytometry for DNA content determination. *B. rotunda* as positive control showed significant inhibitions towards all the cancer cell lines tested with IC₅₀ ranging from 51.0±0.01 to 71.0±1.41 µg mL⁻¹. The remaining *Boesenbergia* species only showed positive cytotoxicity activities against MCF-7 cell lines especially *B. pulchella* var *attenuata* with IC₅₀ value 93.0±2.83 µg mL⁻¹ and *B. armeniaca* with 94.5±0.71 µg mL⁻¹. In cell cycle analysis, *B. rotunda* crude extract arrested cell at sub-G1 phase while *B. pulchella* var *attenuata* arrested cell at G₂/M phase. From this study, it can be concluded that only *B. pulchella* var *attenuata* and *B. armeniaca* were effective against cellular proliferations of MCF-7. However, both species were not as effective as *B. rotunda*.

Key words: *Boesenbergia rotunda*, cancer cell lines, cytotoxicity, IC₅₀, cell cycle

INTRODUCTION

Natural products have been widely used for the treatment of cancer. For the past several decades, natural products were studied intensively as source for anticancer agents (Su and Watanabe, 1993). In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants (Schwartzmann *et al.*, 2002). Many on-going studies were carried out to discover the alternative medicines for the treatment of various acute and chronic diseases

(Seeff *et al.*, 2001; Winslow and Kroll, 1998). Medicinal plants are important for pharmacological research and drug development such as therapeutic agents and as models for pharmacologically active compounds. These contributed to the pharmacology and nutraceuticals industries worldwide.

Boesenbergia belongs to a ginger family, Zingiberaceae in the order of Zingiberales. *Boesenbergia* is a genus of about 80 species, distributed from India to South East Asia. Borneo, as one of the two distribution centres apart from Thailand, which estimated to have 25 species (Larsen, 2003). *Boesenbergia* species is extremely rare compare to other genera. Mostly, they are found in very damp, shaded areas and usually close to streams or in boggy conditions. Thus, there is an urgency to document the plants before facing extinction.

The genus of *Boesenbergia* has attracted more than one specialist in recent years. Many researchers have shown that the rhizome part of *Boesenbergia* spp. displayed health-benefits properties. The *B. rotunda* rhizome has been reported to contain essential oil, boesenbergin, cardamonin, pinostrobin, 5, 7-dimethoxyflavone, 1,8-cineole and panduratin (Kirana *et al.*, 2007). In the primary health care project of Thailand, the rhizome of this plant is used for the treatment of dyspepsia. As regards to its biological activities, *B. rotunda* exhibited antibacterial, antifungal, anti-inflammatory, analgesic, antipyretic, antispasmodic, antitumor and insecticidal activities (Tewtrakul *et al.*, 2003). The rhizome of *B. rotunda* is generally used as a culinary spice in Thailand and also has been used for the treatment of oral diseases (i.e., dry mouth), stomach discomfort, stomach pain, leucorrhoea, diuretic, dysentery, and inflammation. The rhizomes are used in traditional medicine as antiseptic and for the treatment of stomach ache (Hasnah *et al.*, 1995), diarrhea, dermatitis, dry cough and mouth ulcers (Burkill, 1935; Heyne, 1987), gastrointestinal disorders and post-natal treatment (Burkill, 1935). Mahady (2005) reported new *in vitro* and *in vivo* data on two Thai plants from the Zingiberaceae, namely finger-root (*B. rotunda* L Mansf.) and turmeric (*Curcuma longa* L.), both of which are used in Thailand for the treatment of gastrointestinal ailments, including peptic ulcer disease. These antioxidant and anti-inflammatory compounds have often been shown to be effective as anticancer agents.

Therefore, the objectives of the present study were to evaluate the cytotoxicity effects of selected *Boesenbergia* species in Sabah, Malaysia. In this study, *B. rotunda* (available in Peninsular Malaysia) was chosen as positive control.

MATERIALS AND METHODS

Duration and location of study: This study was started from September 2007 until October 2008. All the laboratory works were done at two different places, in which extraction part was done in Universiti Malaysia Sabah (UMS) while anticancer screening and cell cycle analysis were conducted in Universiti Putra Malaysia (UPM). The sample collection were involved in Sabah only with two different areas of Sabah Parks-Tawau Hills Park and Serinsim sub-stesen.

Plant samples collection: Several types of *Boesenbergia* species were collected from Sabah rainforest with permission of Sabah Parks. They were *B. armeniaca*, and *B. pulchella* and *B. pulchella* var. *attenuata*. *B. rotunda* is a cultivated plant and well-known in Peninsular Malaysia as it is sold in wet market such as Chow Kit Market, Kuala Lumpur. *B. pulchella* and its variety as well as *B. pulchella* var *attenuata* were collected in the same conservation area from Tawau that were grown wild in the forests; while *B. armeniaca* was collected from Serinsim.

Voucher specimens of these plants have been deposited at BORNEENSIS, Universiti Malaysia Sabah (BORH) and the Herbarium of the Kinabalu Park, Sabah. They were *B. pulchella* (Ling *et al.*, 2009) *B. pulchella* var *attenuata* (Ling *et al.*, 2009) and *B. armeniaca* (jjLing03). The samples were brought to the Institute for Tropical Biology and Conservation, UMS for further handling for extraction process.

Extractions methodology: According to Wettasinghe *et al.* (2002) with slight modifications, fresh samples (100 g) were sized to *ca.* 1 cm³ dimensions. The samples were frozen at -20°C and lyophilized for 48 h at 13.3 Pa. This lyophilization step was required to afford precise control over the water content of the methanolic extraction medium. The resultant material was ground to a fine powder and 6 g was extracted with 100 mL methanol in an amber container for three days. The residue was twice re-extracted with methanol under the same conditions. The resulting slurry was vacuum-filtered through a Whatman No. 1 filter paper and the filtrate was subjected to vacuum rotary evaporation at 40°C to remove methanol. The concentrated methanolic extract was put in the desiccator until the extract was free from methanol solvent. The extract was stored in the 4°C refrigerator for future usage.

Cytotoxicity study: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, first described by Mossman (1983), is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilization of the cells by the addition of a detergent results in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the formazan product created. The colour can be read in multiwell scanning spectrophotometer (ELISA plate reader).

Culturing of cells: MCF-7 (hormone dependent breast cancer), MDA-MB-231 (non-hormone dependent breast cancer), CaOV₃ and HeLa (cervical cancer), HT29 (colon cancer) cell lines was obtained from American Type Culture Collection (ATCC, USA). 3T3 (mouse fibroblast cell lines) was the normal cell line that was chosen as control. It was cultured in RPMI 1640 medium with L-glutamine. The cells was cultured in the medium supplemented with 10% of fetal calf serum, 1% penicillin streptomycin using 25 cm² flasks in an incubator at 37°C.

MTT assay (Roche Diagnostic, USA): The viability of cells was determined by staining with tryphan blue. Exponential growing cells was harvested and counted by using haemocytometer. The specific medium for that particular cell line was used to dilute the cells to a concentration of 1×10⁵ cells mL⁻¹. From this cell suspension, 100 µL was pipetted into a 96 well microtiter plate and incubated for 24 h in a 5% CO₂ incubator at 37°C. Sample was extracted in a range of doses and added into the plate. After adding the samples extract, new medium was added to make up the final volume of 100 µL in each well. The plate was incubated in a 5% CO₂ incubator at 37°C for 72 h. Then, 10 µL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent was added into each well. This plate was incubated again for 4 h in CO₂ incubator at 37°C.

Subsequently, 100 μL of solubilization solution was added into each well. The cell was then left overnight 37°C , CO_2 incubator. Lastly, the absorbance was read with the ELISA reader at 550 nm.

Cell cycle analysis: Cell cycle was analysed by flow cytometry (FCM) (Model Cyan ADP, Denmark) analysis (Yuan *et al.*, 2004). A total of 2×10^5 cells were harvested from control culture and cells treated with *Boesenbergia* species for 72 h. Cells were washed twice with PBS and fixed in 70% ethanol for 1 h. The samples were then concentrated by removing ethanol. Cellular DNA was stained with 500 μL of 10 $\mu\text{g mL}^{-1}$ propidium iodide in 100 $\mu\text{g mL}^{-1}$ of RNase for 30 min at room temperature, in darkness. Cell cycle distribution was detected with FCM.

Design and statistical analysis: Three species of two different parts were carried out in three replicates. All data represents the results from three independent experiments. Another one species (*B. rotunda*) was designed as control. Analysis of variance was used to test any difference in biological properties resulting from these methods. Duncan test was used to determine significant differences.

RESULTS

Screening for cytotoxicity activity: In the cytotoxicity assay, *B. rotunda* showed the most prominent and promising result as anticancer medicinal plant. It showed positive antiproliferative effect against five cancer cell lines: ovarian (CaOV_3), breast (MDA-MB-231 and MCF-7), cervical (HeLa) and colon (HT-29) cancer cell lines. All of the plant extracts tested solely showed positive results in prohibiting the growth of hormone dependent breast cancer (MCF-7) cell lines (Table 1). The lower the IC_{50} , the more effective the anti-proliferative activity. Vincristine and DL-Sulforaphane were used as positive control. Other than *B. rotunda*, the plant extracts that shown positive result in cytotoxicity assay tested against MCF-7 were the rhizome of *B. pulchella* var *attenuata* and *B. armeniaca*. However, the extracts were less effective than the standards tested in all the cancer cell lines. 3T3 (mouse fibroblast cell lines) is the normal cell line that was chosen as control. This proved that no cytotoxicity was observed in normal cell lines (Data not shown). Figure 1 A and B showed the details of the MTT result of *Boesenbergia* species tested against MCF-7. A dose dependent growth inhibition was observed at concentration ranging from 10 to 100 $\mu\text{g mL}^{-1}$.

Table 1: IC_{50} value of samples tested against several cancer cell lines by using MTT assay

Samples	MCF-7	MDA-MB-231	CaOV_3	HT-29	Hela
<i>B. rotunda</i> (rhizome)	51 \pm 0.01	66.50 \pm 2.12	71.00 \pm 1.41	52.00 \pm 4.24	65.50 \pm 2.12
<i>B. pulchella</i> (rhizome)	ND	ND	ND	ND	ND
<i>B. pulchella</i> (leaves)	ND	ND	ND	ND	ND
<i>B. pulchella</i> var <i>attenuata</i> (rhizome)	93.00 \pm 2.83	ND	ND	ND	ND
<i>B. pulchella</i> var <i>attenuata</i> (leaves)	ND	ND	ND	ND	ND
<i>B. armeniaca</i> (rhizome)	94.50 \pm 0.71	ND	ND	ND	ND
<i>B. armeniaca</i> (leaves)	ND	ND	ND	ND	ND
Vincristine	8.50 \pm 3.41	13.50 \pm 3.14	17.50 \pm 0.82	<1	ND
DL-Sulforaphane	<1	3.0 \pm 0.73	2.50 \pm 2.47	<1	9.50 \pm 5.99

Values are expressed as mean \pm standard deviation ND = Not detected at concentration tested

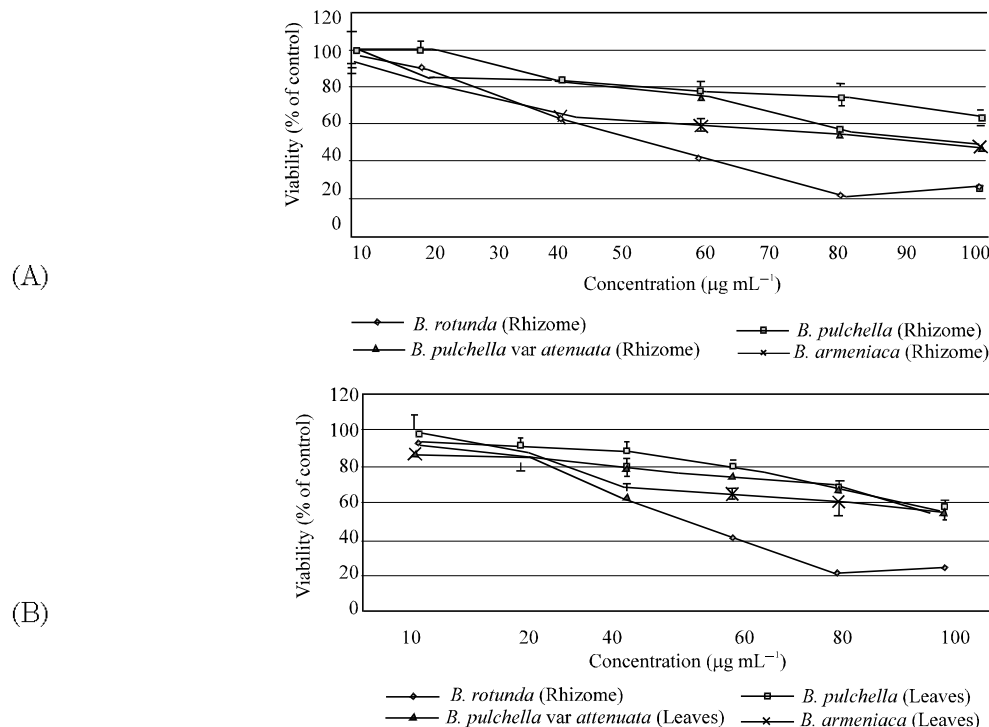


Fig. 1: Growth inhibition of *Boesenbergia* species on breast cancer cell lines (MCF-7) (A) IC_{50} values of MCF-7 determined after 72 h with rhizomes of *Boesenbergia* species; (B) IC_{50} values of MCF-7 determined after 72 h with leaves of *Boesenbergia* species. Optical density (OD) values of each treated group were compared with the control (*B. rotunda*)

Apoptotic cells and cell cycle distribution: The plant extracts that recorded IC_{50} less than $100 \mu\text{g mL}^{-1}$ were proceeded to the next stage: determination of DNA content distribution by using flow cytometry. Cell cycle analyses were done on five different cancer cell lines. Propidium iodide (PI) staining revealed that a similar pattern of DNA content distribution in all cancer cell lines tested. The populations of *B. rotunda* and *B. pulchella* var *attenuata* extracts displayed an obvious increase from sub- G_1 phase, decrease in G_0/G_1 phase, slight increase in S phase and moderate increment in G_2/M phases in MCF-7 compared to control Fig. 2. These indicated that *B. rotunda* and *B. pulchella* var *attenuata* arrested cancer cells at sub- G_1 phase and G_2/M phases respectively. However, MDA-MB-231 showed different DNA content distribution compared to MCF-7. The populations of cells displayed an obvious increase from sub G_1 phase, decrease in G_0/G_1 phase, slight increase in S phase and decrease in G_2/M phases compared to the control Fig. 2A. For CaOV₃, cells distribution revealed an obvious increase from sub- G_1 phase, decrease in G_0/G_1 phase and S phase as well as slight increment in G_2/M phases compared to the control Fig. 2B. For HeLa, the DNA content distribution indicated 60% of apoptosis, which is much higher than control and also shown decrease in G_0/G_1 phase, S phase and G_2/M phases compared to control Fig. 2C. Thus, for MDA-MB-231, CaOV₃ and HeLa, they presented cell arrest at sub- G_1 phase, which indicated apoptosis.

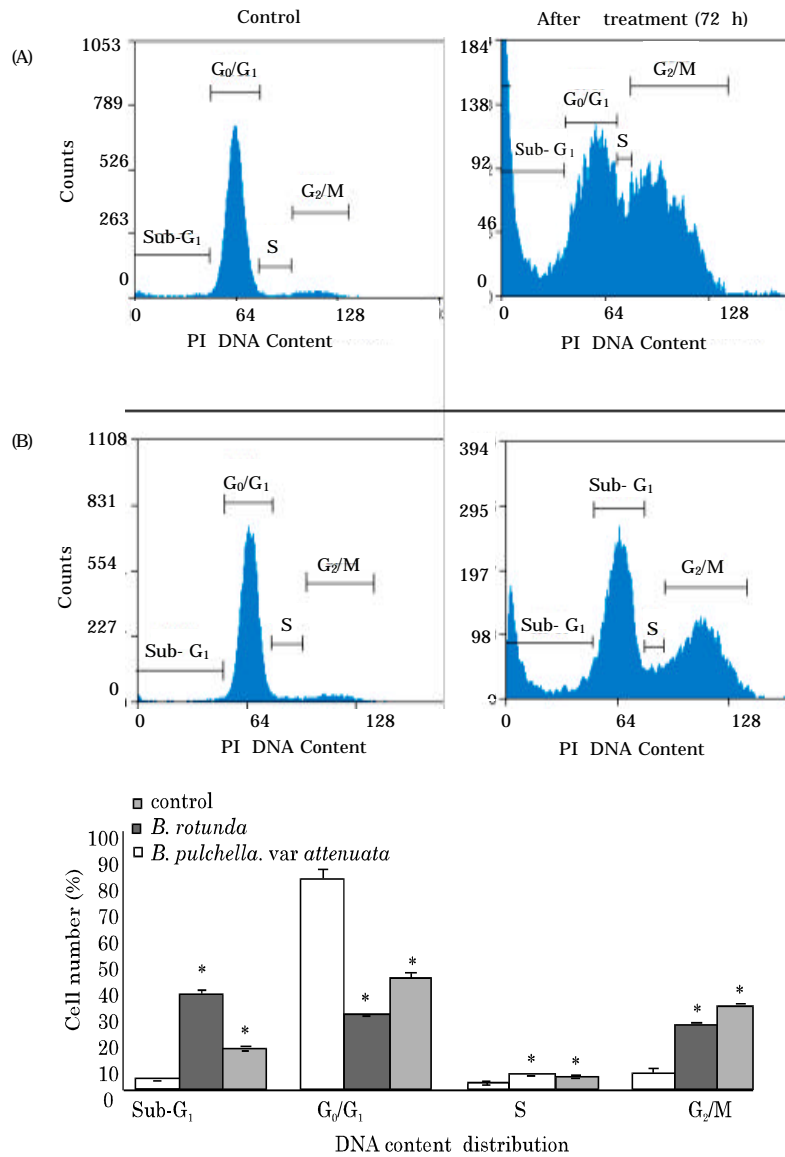


Fig. 2: Apoptotic cells and cell cycle distribution of MCF-7 cells after (A), *B. rotunda* and (B), *B. pulchella* var *attenuata* treatment. Histograms of cell cycle arrest and apoptosis at 72 h. The single asterisk (*) indicates a significant difference from the control ($p < 0.05$)

DISCUSSION

Effectiveness of *boesenbergia* as anticancer agent: *B. rotunda* had been widely known to contain anticancer properties. It has promising anticancer activity towards certain cancer cell lines such as HT29 (colon cancer cells) (Yun *et al.*, 2003) and MCF-7 (hormone dependent breast cancer cells) (Kirana *et al.*, 2007), exhibited anti-HIV-1 protease inhibition (Tewtrakul *et al.*, 2003), inhibited dengue-2 virus NS3 protease (Tan *et al.*, 2006) and displayed chemopreventive against gastric cancer (Censini *et al.*, 1996).

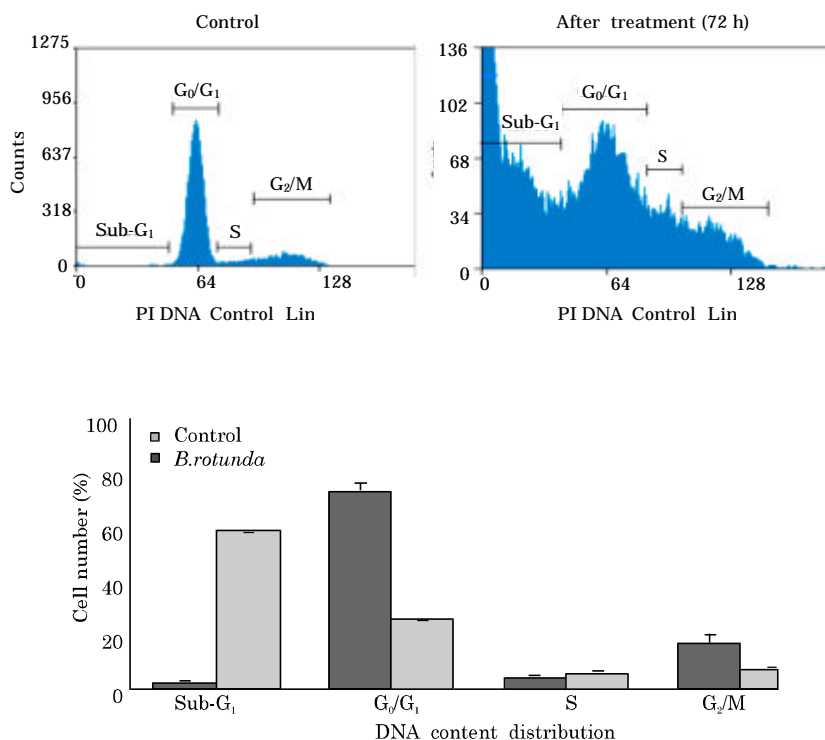


Fig. 2A: Apoptotic cells and cell cycle distribution of MDA-MB-231 cells after *B. rotunda* treatment. Histograms of cell cycle arrest and apoptosis at 72 h

Most *Boesenbergia* species were found to contain flavonol compounds such as flavone glycosides, isoflavone and chalcone. The *B. rotunda* rhizome has been reported to contain essential oil, boesenbergin, cardamonin, pinostrobin, 5, 7-dimethoxyflavone, 1,8-cineole and panduratin. Panduratin A was found to inhibit the growth of MCF-7 human breast cancer and HT-29 human colon adenocarcinoma cells with an IC_{50} of 3.75 and 6.56 $\mu\text{g mL}^{-1}$, respectively. Panduratin A arrested cancer cells labelled with Annexin-V and propidium iodide in the G_0/G_1 phase and induced apoptosis in a dose-dependent manner (Kirana *et al.*, 2007).

In the present study, only *B. rotunda* (rhizome) showed positive cytotoxicity for the entire five cancer cell lines tested (Table 1 and Fig. 1). It was discovered that the leaves part of all *Boesenbergia* species did not showed positive cytotoxicity at the concentration tested. The cancer cell growth inhibition was in dose dependent manner (Fig. 1). Therefore, the leaves part might need higher doses compared with rhizomes to exhibit cytotoxic effects. These concluded that rhizome part of *Boesenbergia* is better as antiproliferative agent than leaves against cancer cell lines. Furthermore, the rhizome extracts of *B. rotunda*, *B. pulchella* var *attenuata* and *B. armeniaca* have been shown to induce cytotoxic activity in breast cancer cell line (MCF-7) with IC_{50} values of 51 ± 0.01 , 93 ± 2.83 and 94.5 ± 0.71 $\mu\text{g mL}^{-1}$, respectively (Table 1), even though antioxidant assays as well as total phenolic and flavonoid content conducted in previous study did not show better results for *B. rotunda* and *B. armeniaca* when compared to other *Boesenbergia* species (*B. pulchella* and *B. pulchella* var *attenuata*) (Ling *et al.*, 2009).

The antioxidant activities of *Boesenbergia* species were probably a reflection of their phenolic content in which the antioxidant properties of phenolic compounds were contributed

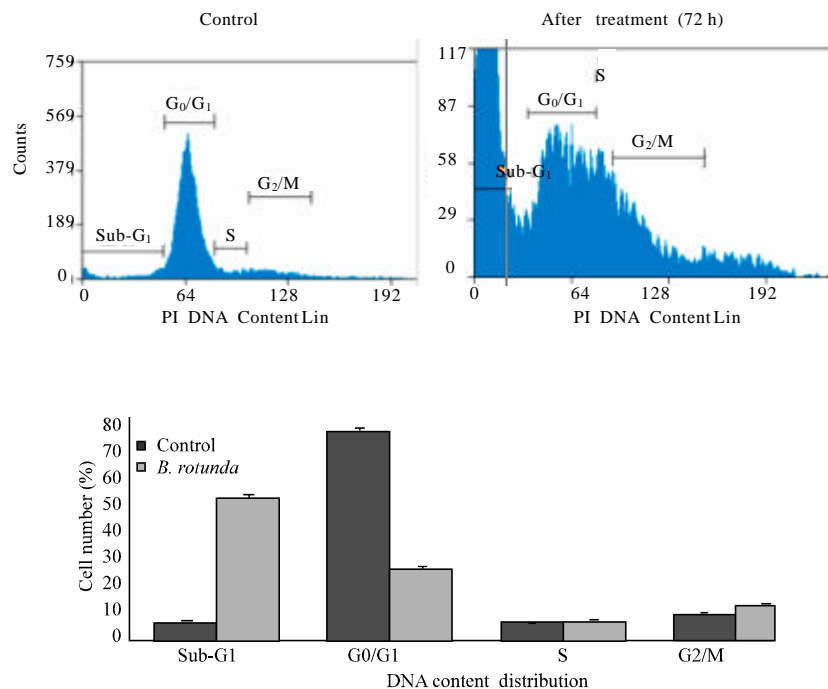


Fig. 2B: Apoptotic cells and cell cycle distribution of CaOV₃ cells after *B. rotunda* treatment. Histograms of cell cycle arrest and apoptosis at 72 h

by the reactive phenol moiety (hydroxyl group on aromatic ring), which has the capability to scavenge free radicals via hydrogen or electron transfer. Both ascorbic acid and total phenolics showed high positive correlation with antioxidant activity measured in methanol extract as determined by all assays, which indicates that ascorbic acid and total phenolics are important contributors to antioxidant activity in *Boesenbergia* extracts. This was in the agreement of Thaipong *et al.* (2006), Sun and Ho (2005) and Abu-Bakar *et al.* (2009).

High antioxidant level did not necessarily reflect the high anticancer effect of the plant extract. These might be due to synergistic effect of the crude extract (Ling, 2010). In our previous study, leaves part showed higher antioxidant properties than its rhizome part. This was in the agreement of Herrmann (1988). It depends on the presence of phytochemicals or certain bioactive compound that responsible for the antioxidant and antiproliferative activity (Abu-Bakar *et al.*, 2010).

In the family of Zingiberaceae, it is generally believed that antioxidants produced by the plants are transported to the rhizome where they are accumulated. This implies that rhizomes would contain higher phytochemicals than other plant parts (Chan *et al.*, 2008).

However, it depends on the habitat and exposure of sunlight (Masuda *et al.*, 1999). Foliage of tropical forest plants produces more antioxidants when exposed to elevated light conditions (Frankel and Berenbaum, 1999). For example, *Etilingera* species are the largest of the ginger plants and can grow up to 6 m in height, have the highest total phenolics content and antioxidant activity (Khaw, 2001). *Boesenbergia* genus is normally available at damp and shaded areas in the Borneo, with height of 20-60 cm. This special habitat restricted it from exposing to sunlight. This could explain that the species has lower antioxidant activity compared to other genera in Zingiberaceae.

The key factor contributing to these anticancer activities might be the active compounds presented in the different parts of the plants. The useful active components which inhibit the

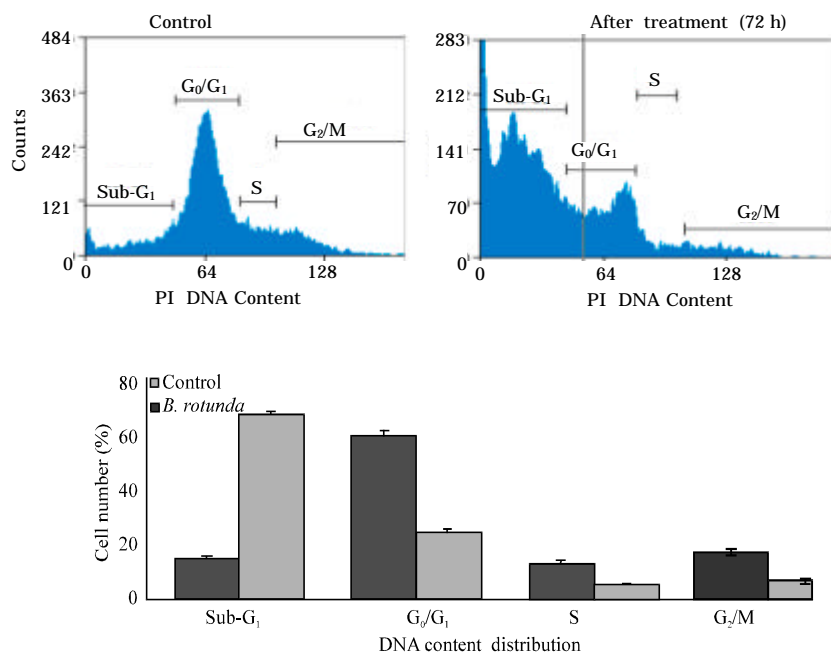


Fig. 2C: Apoptotic cells and cell cycle distribution of HeLa cells after *B. rotunda* treatment. Histograms of cell cycle arrest and apoptosis at 72 h

carcinoma cells found in rhizome part may not be found in the leaves. Therefore, some of the results showed negative inhibition of the cancer cell lines. It was probably the active compounds of the plants, which may act as an antioxidant, vary according to the different parts of the plants.

Ling *et al.* (2009) has discovered that quercetin and luteolin might be the agent possible contributed to anticancer activities in *B. rotunda* and *B. armeniaca* respectively. It was because quercetin was the major flavonols presented in *B. rotunda*, while luteolin was the major flavones in *B. armeniaca*. Moreover, Huang *et al.* (1999) discovered that quercetin and luteolin induced apoptosis in a wide range of tumor cells such as Hep G₂ and MCF-7. Wei *et al.* (1994) reported that quercetin induced apoptosis, characterized by typical morphological changes, in certain tumor cell lines. These had proven that the two flavonoids type contributing to anticancer activities in this study.

As a consequence, these species may become promising potential as anticancer agent to combat hormone dependent breast cancer (MCF-7). The result was in agreement with Kirana *et al.* (2007) as *Boesenbergia* plant extracts were more sensitive to MCF-7 breast cancer than HT-29 colon cancer cells.

In conclusion, *B. pulchella* var *attenuata* and *B. armeniaca* were potent anticancer remedy especially for suppressing hormone dependent breast cancer (MCF-7). In order to further confirm the function of *B. rotunda* and *B. pulchella* var *attenuata* extracts in inducing cell apoptosis, a more definitive study using double cell labeling (e.g. Annexin V-PE and 7-AAD) will be performed in future. The potential medicinal uses of these *Boesenbergia* species are supported by the presence of antioxidant (Ling *et al.*, 2009) and anticancer activities. Hence, the need to exploit the potentials of these plants especially in areas of traditional medicine and pharmaceutical industries arises.

ACKNOWLEDGMENTS

Authors would like to extend their appreciation to Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah; Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM); Institute Bioscience, UPM for the use of laboratory facilities and technical assistance as well as Sabah Parks for the permission of sample collection.

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