

## Crude Extracts of *Agaricus brasiliensis* Induce Apoptosis in Human Oral Cancer CAL 27 Cells through a Mitochondria-dependent Pathway

MING-JEN FAN<sup>1</sup>, YU-CHIN LIN<sup>1</sup>, HSIN-DER SHIH<sup>2</sup>, JAI-SING YANG<sup>3</sup>, KUO-CHING LIU<sup>4</sup>, SU-TSO YANG<sup>5</sup>, CHIEN-YIH LIN<sup>1</sup>, RICK SAI-CHUEN WU<sup>6</sup>, CHUN-SHU YU<sup>7</sup>, YANG-CHING KO<sup>8</sup> and JING-GUNG CHUNG<sup>1,9</sup>

<sup>1</sup>Department of Biotechnology, Asia University, Taichung 413, Taiwan, R.O.C.;

<sup>2</sup>Plant Pathology Division, Taiwan Agricultural Research Institute, Council of Agriculture, Taichung 413, Taiwan, R.O.C.;

Departments of <sup>3</sup>Pharmacology, <sup>9</sup>Biological Science and Technology, and <sup>4</sup>Medical Laboratory Science and Biotechnology,

<sup>7</sup>School of Pharmacy, China Medical University, Taichung 404, Taiwan, R.O.C.;

<sup>5</sup>Department of Radiology, China Medical University Hospital and School of Chinese Medicine, China Medical University, Taichung 404, Taiwan, R.O.C.;

<sup>6</sup>Department of Anesthesiology, China Medical University Hospital, Taichung 404, Taiwan, R.O.C.;

<sup>8</sup>Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, St. Martin De Porres Hospital, Chiayi 600, Taiwan, R.O.C.

**Abstract.** In Taiwan, oral cancer is the fourth leading cause of male cancer mortality, and is still increasing. The Basidiomycete, *Agaricus brasiliensis* Murill (ABM) is a dietary mushroom and has been known for its immune-enhancing, antitumor, antioxidation, antiviral and antimutagenesis functions. However, the exact anticancer mechanisms of ABM on human oral cancer cells are still unclear. In the present study, we investigated the effects of 50% ethanol crude extracts and hot water extracts of ABM on oral cancer CAL 27 cells. We observed that 0.9 mg/ml and 0.7 mg/ml of ABM 50% ethanol crude extracts and hot water, respectively, caused morphological changes and significantly reduced cell viability after 48-h treatment. The results showed that both extracts of ABM inhibited cell proliferation, increased the Ca<sup>2+</sup> release, reduced the mitochondria membrane potential ( $\Delta\Psi_m$ ), and caused cell cycle arrest in the G<sub>0</sub>/G<sub>1</sub> phase, which contributed to apoptosis. Additionally, ABM induced DNA fragmentation, a characteristic of apoptosis and the expressions of apoptosis-related proteins, including apoptosis-inducing factor, cytochrome c, and caspase-3, were increased. Overall, we demonstrated that 50%

ethanol crude extract and hot water extracts of ABM were able to induce apoptotic cell death in CAL 27 cells via the release of cytochrome c from mitochondria into the cytoplasm and activation of caspase-3 *in vitro*.

*Agaricus brasiliensis* Murill (ABM) is an edible mushroom that has recently been used as health supplement in Asian countries, including Taiwan and China. This mushroom is also widely produced in Brazil, Japan and Korea, where it is consumed as food and tea for its medicinal effects (1). ABM has been reported to contain chemical components, including protein-bound and free polysaccharides (2, 3), and steroids (4), which have been widely studied for antitumor activity (5-9) or immunomodulation (4, 10-13). (1-4)-D-Glucan with (1-6)- $\beta$  branching from ABM crude extract has been reported to activate natural killer (NK) cells and induce apoptosis (7, 14-15). Many reports have shown that ABM possesses antitumor activity (2, 8). Additional biological activities of ABM, such as sterilization (16), anti-angiogenic (17), anti-diabetic (18), antioxidant (19, 20), and antimutagenity (21-23) activities have been reported (24).

Head and neck squamous cell carcinomas (HNSCC) have broadly varying incidence and mortality rate around the world, particularly in Southeast Asia and Eastern Europe (25-26). Cancer of the mouth and pharynx is the sixth most common cancer worldwide. Over 90% of these oral-pharyngeal tumors are squamous cell carcinomas (SCC) (27). Oral cavity cancer is the leading cause of death from head and neck cancer (28). In Taiwan, HNSCC is the sixth most common cancer according to the Department of Health,

Correspondence to: Jing-Gung Chung, Department of Biological Science and Technology, China Medical University, No. 91, Hsueh-Shih Road, Taichung 40402, Taiwan, R.O.C. Tel: +886 422053366 ext. 2161, Fax: +886 422053764. e-mail: jgchung@mail.cmu.edu.tw

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R.O.C. (Taiwan). In Western countries, there is convincing evidence that a large attributable risk can be ascribed to the joint habits of cigarette smoking and alcohol consumption (29). In Asian societies, a high attributable risk can be ascribed to cigarette smoking and areca nut chewing (29).

Many reports have demonstrated that mitochondria-mediated apoptosis is regulated by the BCL-2 family of proteins, which can promote (BAX and BID) or inhibit (BCL-2 and BCL-X<sub>L</sub>) apoptosis (6-7). BCL-2 is especially important for the preservation of the outer mitochondrial membrane, thereby preventing the release of pro-apoptotic factors from the mitochondria (8, 9). Overexpression of BCL-2 apparently inhibits induction of apoptosis in response to a variety of chemical agents through inactivation of caspase-3 and poly(ADP-ribose) polymerase (PARP) degradation (4, 10). Although the effects of BCL-2 on the release of pro-apoptotic factors have been described in detail, the role of BCL-2 in ABM-mediated apoptosis is not clear. The AKT pathways are highly ubiquitous and mediate important regulatory signals within the cell. AKT has been implicated in cell proliferation, regulation of apoptosis, and angiogenesis (11-12).

In this study, we examined the effects of crude extracts of ABM on oral cancer CAL 27 cells, studying the regulation of cell proliferation, cell cycle progression and apoptosis. Although ABM has been reported to possess medicinal qualities, the cellular and molecular mechanisms underlying ABM-induced apoptosis are not clear.

## Materials and Methods

*Crude extracts of A. brasiliensis Murill (ABM).* *A. brasiliensis*, strain L4, was planted at our contracted mushroom producer located in Wufeng, Taichung, Taiwan. These mushrooms were cultivated in a heavy metal contamination-free protocol and fruiting bodies were found to be free of Cr, Hg, and Pb content after harvest. The fresh fruit was desiccated and ground to 50 mesh powder. To obtain aqueous (hot water) or ethanol (50%) extracts of the basideocarp, 50 g of the dry basideocarp were resuspended in 100 ml of distilled water or 50% ethanol, at ambient temperature (approximately 25°C), under agitation for 1 h (30). The crude extract of ABM was prepared as described: hot water or 50% ethanol extract was used in the form of aqueous extract (2.5%) prepared at 100°C for 30 min (hot water) or room temperature for 30 min (50% ethanol), resuspended three times. All the solutions were filtered through cellulose ester membrane with 0.22 mm pore, followed by ultra-low temperature dehydration to powder. The prepared ABM was a greenish-brown powder that was subsequently dissolved in phosphate-buffered saline (PBS), filtered through a 0.22 mm filter, made up to 100 mg/ml and stored at -20°C (31-32).

*Cell line and culture conditions.* The human oral cancer cell line (CAL 27) was purchased from Food Industry Research Development Institute (Hsinchu, Taiwan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco BRL/Invitrogen, Carlsbad, CA, USA),

2 mM L-glutamine, 100 Units/ml penicillin and 100 µg/ml streptomycin (Gibco BRL/Invitrogen). The cells were cultured in a Petri dish at 37°C, 5% CO<sub>2</sub>, RH 95%. After the third subculture, these cells were cultured in 12-well, 24-well and 96-well cell culture plates and 75T cell culture bottles for further research.

*Cell viability and morphology determinations.* The cells were seeded at a density of 1×10<sup>4</sup> cells/100 µl culture medium in 96-well plates and then incubated under various conditions as specified in the text. About 100 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml; Sigma-Aldrich Corp., St. Louis, MO, USA) was subsequently added to each well. After 3 h of additional incubation, one hundred µl of a solution containing 10% sodium dodecyl sulfate (SDS, pH 4.8) plus 0.01 N HCl was added to dissolve the crystals. The absorption values at 570 nm were determined with an ELISA plate reader (33). For the morphological study, cells were grown on 12-well plates and then were treated with ABM for 24 h and were photographed under a phase-contrast microscope (34).

*DNA content by flow cytometric analysis.* Cells were washed with PBS, pelleted by low-speed centrifugation and fixed in 70% ethanol at -20°C overnight. Subsequently, nuclei were stained with propidium iodide (PI) solution in PBS containing 40 µg/ml PI, 0.1 mg/ml RNase A and 0.1% Triton X-100 in a dark room for 30 min at 37°C. The DNA content in each cell nucleus was determined with a FACSCalibur flow cytometer (35, 36).

*DNA gel electrophoresis for apoptotic cells.* A total of 1×10<sup>6</sup> cells/well of CAL 27 cells in 6-well plates were exposed to ABM for 24, 48 and 72 h and then cells were harvested and lysed in a digestion buffer containing 0.5% sarkosyl, 500 µg/ml proteinase K, 50 mM Tris-HCl (pH 8.0) and 10 mM EDTA at 55°C for 12 h. Cells were treated with 1 µg/ml RNase A for 2 h at 37°C. DNA was extracted by phenol-chloroform-isoamyl alcohol extraction (25:24:1) as previously described (37). The extracted DNA was resuspended and loaded in each well, and DNA gel electrophoresis was performed using 2% agarose gel. After ethidium bromide staining, the DNA ladders were photographed under UV light as previously described (37-38).

*4',6-Diamidino-2-phenylindole (DAPI) nuclear staining.* The examined CAL 27 cells were washed with cold PBS and fixed with 3.7% (w/v) paraformaldehyde in PBS for 10 min at room temperature. After permeabilization, the cells were stained with a DAPI (10 µg/ml) solution at 37°C for 30 min. The cells were washed with PBS and were examined and photographed using a fluorescence microscope (39-40).

*Assays for mitochondrial membrane potential ( $\Delta\Psi_m$ ), Ca<sup>2+</sup> levels and reactive oxygen species (ROS).* CAL 27 cells (2×10<sup>5</sup>/well) were plated onto 12-well plates and treated with ABM for 3, 6, 12 24 or 48 h before being harvested, washed twice, and re-suspended in a solution containing the mitochondrial membrane potential indicator 3,3'-dihexyloxacarbocyanine iodide (DiOC6, 1 µM), Ca<sup>2+</sup> probe Fluo-3/AM (2.5 µg/ml) and ROS indicator 2,7-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF-DA, 5 µM), and then incubated at 37°C for 30 min. The changes of fluorescence intensity in  $\Delta\Psi_m$ , Ca<sup>2+</sup> levels and ROS were determined by flow cytometry and analyzed by BD CellQuest Pro software as previously described (41, 42).

**Western blotting analysis.** CAL 27 cells ( $1 \times 10^6$  cells/well) were plated in 6-well plates and ABM was added to cells in each well for 12, 24, 48 or 72 h. Cells in each well were harvested and resuspended in ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM EDTA and 0.1% Triton X-100. The collected cells were centrifuged at  $13,000 \times g$  for 10 min at  $4^\circ\text{C}$  to remove cell debris and the supernatant collected for determination of total protein concentration as described previously (39). SDS gel electrophoresis and Western blotting were performed as described previously (39, 42) for determining the effects of ABM on protein levels of cell cycle-regulated and apoptosis signals.

**Statistical analysis.** All experiments were performed in triplicate. The results of multiple observations are presented as the mean  $\pm$  S.D. of at least three separate experiments. Statistical significance was determined using Student's *t*-test. A value of  $p < 0.05$  was considered to represent a statistically significant difference.

## Results

**Effects of crude extract of ABM on cell viability of CAL 27 cells.** In the present study, we investigated the effect and mechanism of 50% ethanol and hot water crude extracts of ABM on the viability of CAL 27 cells by MTT assay. It was shown that 0.25-2 mg/ml crude extracts of ABM caused morphological changes and significantly reduced cell viability at 72-h treatment in CAL 27 cells (Figure 1A). CAL 27 cells showed significant decrease in their viability with increasing concentration of extract and duration of treatment (Figure 1A and B). By integrating these data, the  $\text{IC}_{50}$  for EtOH 50% extract and hot water extract were found to be 0.9 mg/ml and 0.7 mg/ml, respectively. It was shown that 50% EtOH and hot water crude extracts of ABM were able to induce cytotoxicity in CAL 27 cells. These effects are time and dose dependent. The morphological study suggests that crude extracts of ABM can trigger cell death with features of apoptosis in CAL 27 cells (Figure 2A and B). After a 48-h treatment with ABM crude extract, even at low concentration (0.5 mg/ml), cell morphology became abnormal with cell membrane shrinkage, and the cell numbers were significantly less than these of control treatment (Figure 2B).

**Effects of crude extract of ABM on cell cycle progression in CAL 27 cells.** The ratio of cells in the  $\text{G}_0/\text{G}_1$  phase was increased after 48 h of ABM treatment, and the cell cycle arrested at the  $\text{G}_0/\text{G}_1$  phase (Figure 3).

**Effects of crude extract of ABM on DNA fragmentation and apoptosis in CAL 27 cells.** By using gel electrophoresis, DNA showed significant ladders at 180-200 bp in CAL 27 cells after treatment with ABM extracts (Figure 4). By using PI staining and flow cytometric assay, with increasing treatment time, CAL 27 cells treated with 0.9 mg/ml EtOH crude extract showed increased percentage of sub- $\text{G}_1$  stage cells and

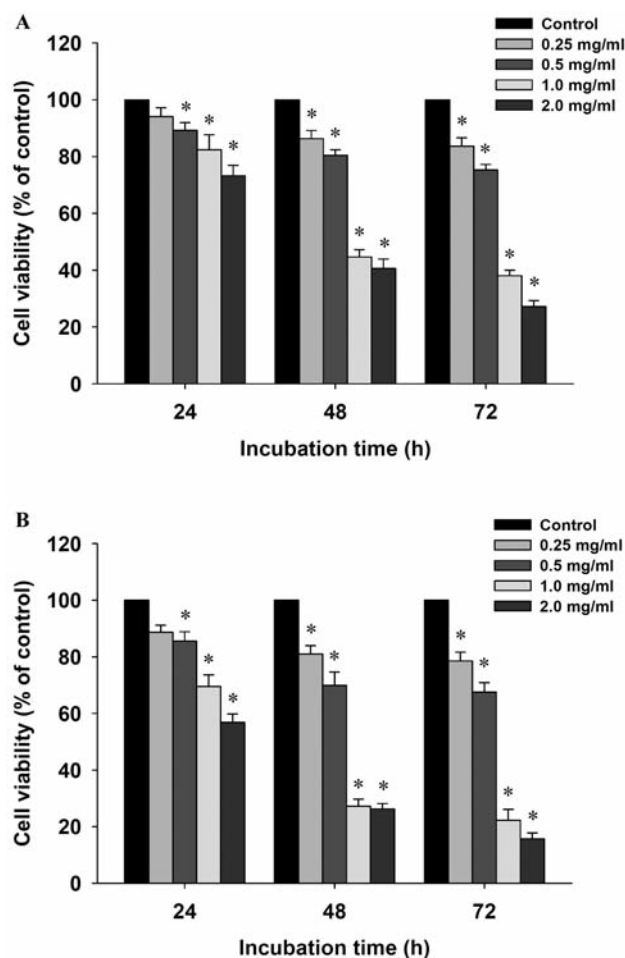


Figure 1. 50% EtOH (A) and hot water (B) crude extracts of *Agaricus brasiliensis* Murill (ABM) affect cell viability in human oral cancer CAL 27 cells. Cells were treated with different doses of ABM crude extracts for 24, 48 and 72 h for cell viability examinations as described in the Materials and Methods. Each point is mean  $\pm$  S.D. of three experiments. \* $p < 0.05$  compared to control (0  $\mu\text{g/ml}$  ABM).

the cell number in  $\text{G}_0/\text{G}_1$  phase increased (Figure 3). After a 48-h treatment, the number of cells in the  $\text{G}_2/\text{M}$  phase was near zero (Figure 3). The DNA extracted from these cells showed significant fragmentation after 48-h and 72-h treatment with ABM 50% EtOH or hot water extracts (Figure 4A). The results from MTT assay, flow cytometry and DNA gel electrophoresis showed that CAL 27 cells treated with 0.9 mg/ml 50% EtOH crude extract of ABM demonstrated inhibition of cell proliferation, arrest of cell cycle in  $\text{G}_0/\text{G}_1$  phase, DNA damage and commitment to apoptosis (Figure 4A and B). The CAL 27 cells treated with 0.7 mg/ml hot water crude extract also exhibited the same effect (Figure 4A and B). The morphologies of CAL 27 cells treated with ABM determined by DAPI staining and then observed under fluorescence microscopy confirmed these results (Figure 4B).

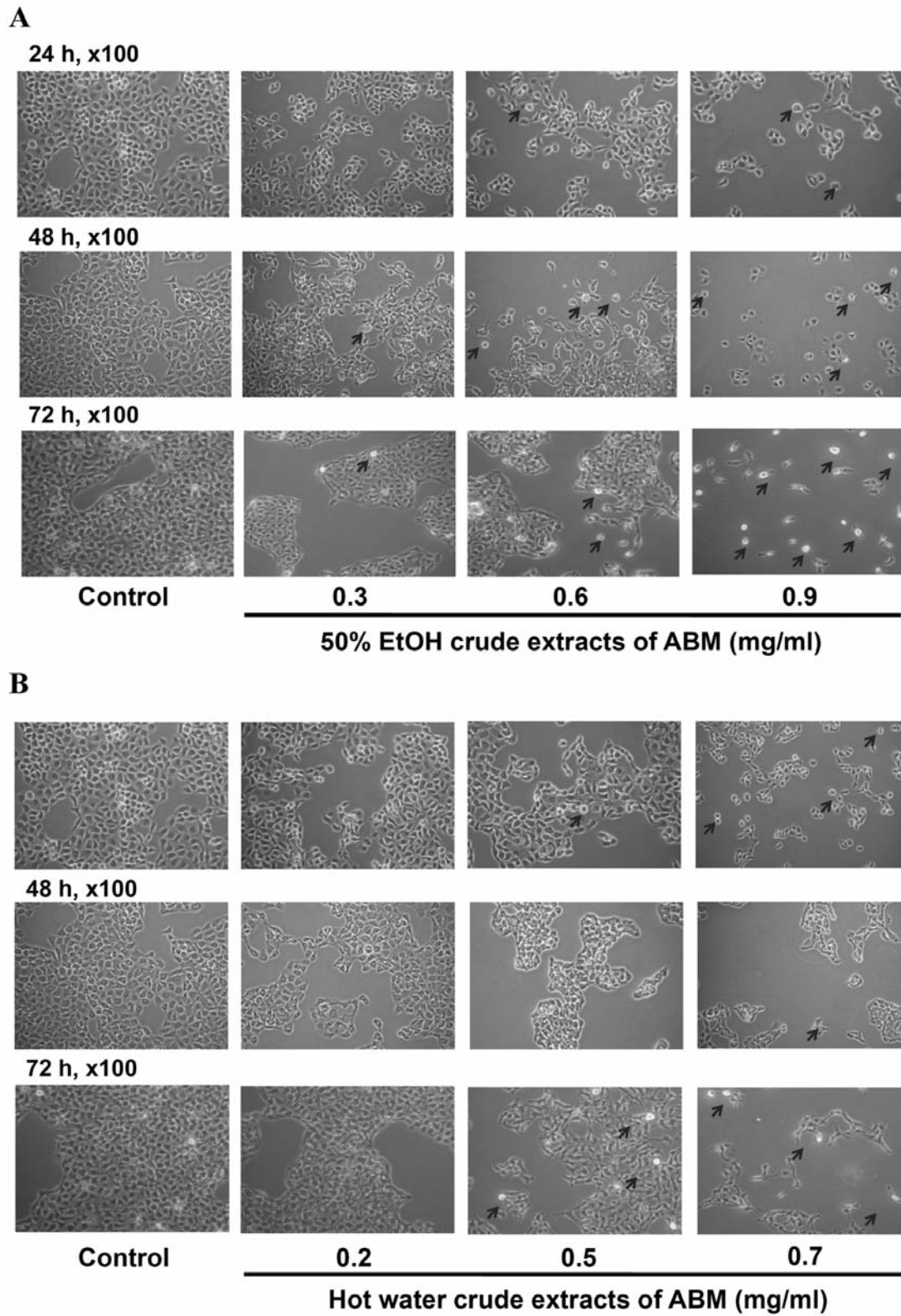


Figure 2. The morphological change of CAL 27 cells after treatment with different concentrations of 50% EtOH (A) and hot water (B) crude extracts of ABM. Cells were placed in 12-well plates and treated with 50% EtOH (A) or hot water (B) crude ABM extracted for 24, 48 and 72 h. Cells were examined and photographed under a phase-contrast microscope. The arrows indicate cell death with apoptotic features.

Compared to the controls, with time after treatment with ABM EtOH and hot water extracts, fluorescence increased and cell numbers were reduced due to nuclear shrinkage, chromosome condensation and apoptotic bodies, and the number of apoptotic cells increased.

**Effects of crude extract of ABM on mitochondrial membrane potential ( $\Delta\Psi_m$ ),  $Ca^{2+}$  and ROS of CAL 27 cells.** MTT assay and DNA fragmentation analysis showed that ABM extracts caused cell cycle progression and apoptosis of CAL 27 cells. Assays of mitochondria membrane electronic potential and  $Ca^{2+}$  release were carried out to determine whether the mitochondria play a role in the control of apoptosis of treated cells (43, 44). The  $\Delta\Psi_m$  was assayed by flow cytometry using DiOC6 fluorite dye which binds to the mitochondrial membrane (45). The results showed a curve shift with treatment time to left. The  $\Delta\Psi_m$  was altered especially after treatment with crude ABM extracts for 24 h which might cause apoptosis of cells (Figure 5A and B). The  $Ca^{2+}$  release also showed the same results. The  $Ca^{2+}$  release correspond to the time of treatment with 50% EtOH (Figure 5C) and hot water (Figure 5D) extracts of ABM in CAL 27 cells. After ABM treatment, the  $Ca^{2+}$  was released into the medium and slowly increased with the time, becoming significant after 12 and 24 h for EtOH extract treatment (Figure 5C). For hot water extract, the response of  $Ca^{2+}$  release was quicker (Figure 5B) and was significant at all times measured. The ROS production of CAL 27 cells after exposure to ABM extracts was also assayed by  $H_2DCF$ -DA. The results of flow cytometry showed that for cells treated with ABM extracts, the peak was significantly shifted to the left which means the ROS did not increase after ABM treatment (Figure 5E and F), but in fact was significantly reduced. The 50% EtOH (A) and hot water (B) crude extracts of ABM had similar effects. Therefore, ROS are not likely to play a key factor in mitochondria-mediated apoptotic cell death.

**Effects of ABM on cell cycle and apoptosis-associated protein expression in CAL 27 cells.** Proteins related to cell progress were assayed by Western blotting in CAL 27 cells treated with ABM extracts. Proteins extracted from CAL 27 cells which were treated with 0.9 mg/ml 50% EtOH or 0.7 mg/ml hot water ABM extracts, after 24, 48 and 72 h, were subjected to Western blotting assay to measure the change of apoptosis-related proteins. The results showed that proteins related to the cell cycle such as p53, p21 and p27 were increased after ABM treatment, while CHK1 increased initially but decreased after 72 h compared to 0 h treatment (Figure 6A). The amount of cyclin proteins such as cyclin D, CDK4, and cyclin E proteins were decreased but cyclin A and CDK2 were increased after ABM treatment compared to untreated cells (Figure 6B). This indicates that ABM extracts induced cell cycle arrest in  $G_0/G_1$  phase in CAL 27 cells.

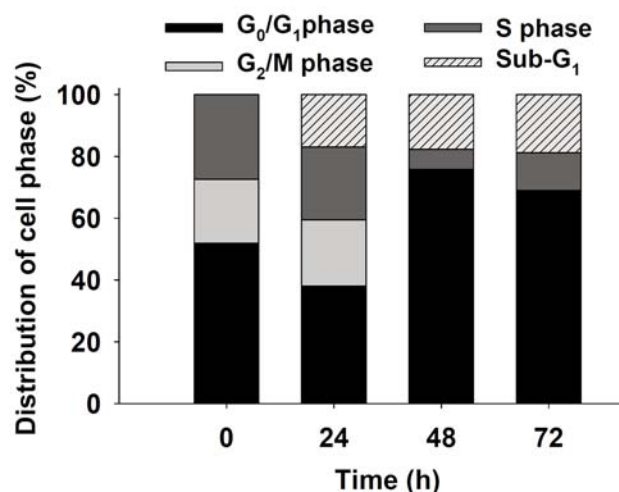


Figure 3. Effects of 50% EtOH crude extracts of ABM on the cell cycle in human oral cancer CAL 27 cells. Cells treated with 0.9 mg/ml EtOH crude extract of ABM showed accumulation in the sub- $G_1$  stage in proportion to treatment time. After a 48-h treatment, the proportion of cells at  $G_2/M$  phase was near zero, indicating arrest in  $G_0/G_1$  phase.

The results also showed that cell apoptosis protein BAX (Figure 7A) increased. The protein levels of cytochrome *c* and AIF released from mitochondria also increased with time (Figure 7B and C). These proteins related to downstream in cell apoptosis, caspase-3 and PARP, also increased after ABM treatment (Figure 7B). The 0.7 mg/ml hot water extracts also had similar results; expressions of BAX, cytochrome *c*, AIF and caspase-3 were increased after ABM treatment (Figure 7D), while that of PARP decreased (Figure 7D), causing loss of DNA repair mechanism and inducing cell apoptosis.

## Discussion

ABM is a basidiomycete, native to south-eastern Brazil, which has been frequently used as a health supplement, mainly in the form of tea, to combat various symptoms (physical and emotional stress, high cholesterol levels, diabetes, etc.), but also in experimental cancer treatment (46). The fruiting body of *A. brasiliensis* consists of 85-87% water. It is rich in protein and also contains carbohydrates, dietary fiber, lipids, and vitamins, especially B1, B2 and niacin. Ergosterol and linoleic acid are the predominant lipids. Aromatic, non-volatile compounds such as the common soluble sugars arabinose, glucose and trehalose are also found (2-4). Potassium is the main mineral component in *A. brasiliensis* (47). The extract of the fruiting bodies of *A. brasiliensis*, contains a  $\beta$ -D-glucan-protein complex consisting of 50% carbohydrate and 43% protein, which was found to contain large amounts of alanine (11%) and tyrosine

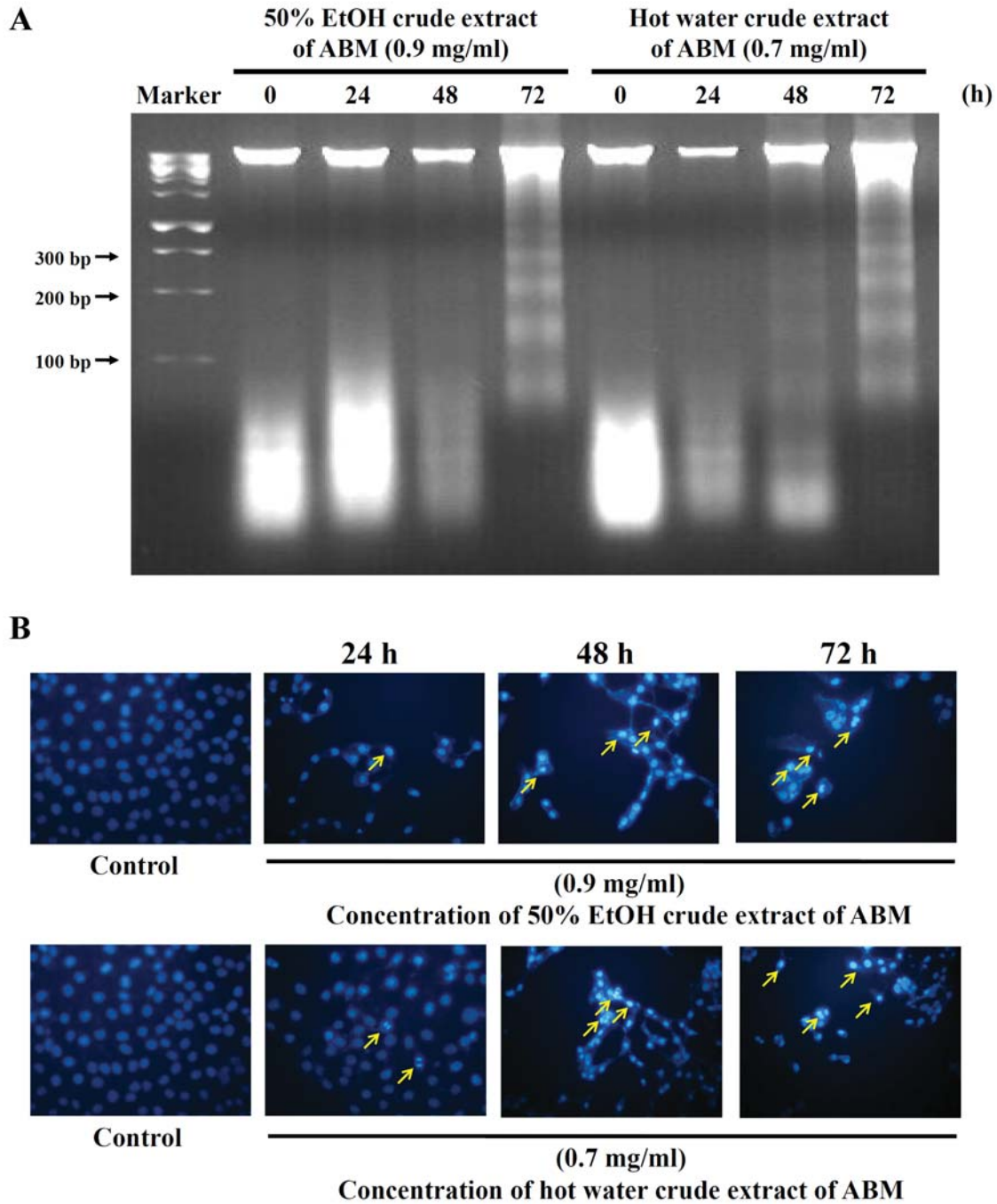


Figure 4. Effect of 50% EtOH and hot water crude extracts of ABM on the DNA fragmentation and apoptosis in human oral cancer CAL 27 cells. Cells were placed in 6-well plates and then treated with various doses of 50% EtOH (A) and hot water (B) extracts of ABM for 0, 24, 48 and 72 h. Cells were harvested and DNA was isolated for DNA gel electrophoresis. ABM-induced cell death for 0-72 h in CAL 27 cells was estimated by DAPI staining. After 48 and 72 h ABM treatment, fluorescence increased compared to the control. The arrows show nuclear condensation and apoptotic cells.

(2-4%). *A. blazei* also contains a  $\beta$ -D-glucan polysaccharide, but with  $\beta$ -(1,6) linkages (48).  $\beta$ -1-6-D-Glucan is the main compound of *A. brasiliensis* and is currently associated with antitumor activity (7, 14).

In the present study, the extracts prepared using two different methods (50% EtOH and hot water) did not present genotoxic activity in CAL 27 cells under the conditions tested. However, Mizuno *et al.* (46) emphasized that basidiomycete properties

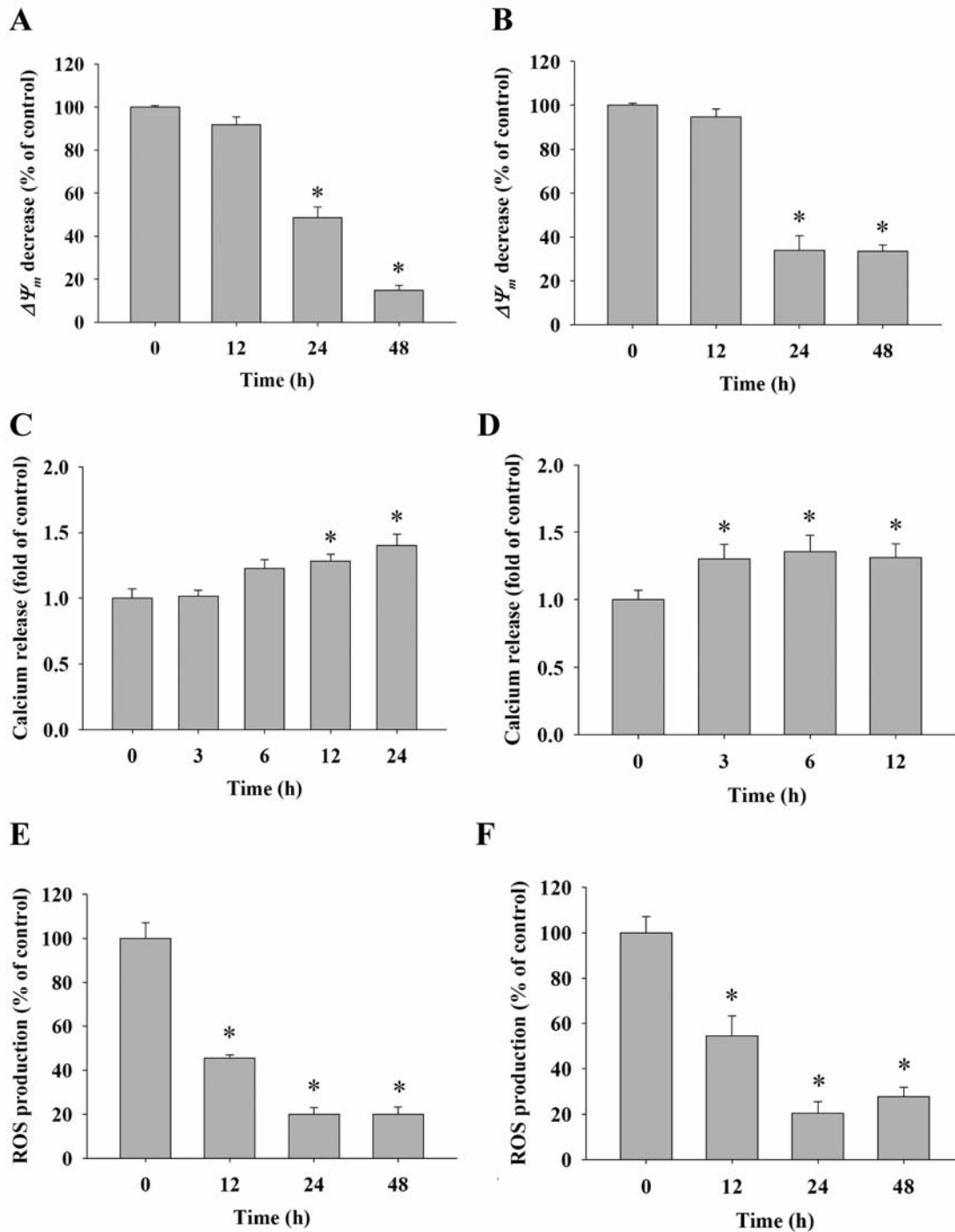


Figure 5. Effect of 50% EtOH and hot water crude extracts of ABM on mitochondria membrane potential ( $\Delta\Psi_m$ ),  $Ca^{2+}$  and reactive oxygen species (ROS) in CAL 27 cells. Cells were treated with 50% EtOH (A, C and E) and hot water (B, D and F) crude extract ABM for 0, 12, 24 and 48 h, and then harvested for staining by DiOC6, Fluo-3/AM and DCFH-DA for  $\Delta\Psi_m$ ,  $Ca^{2+}$  and ROS, respectively. \* $p < 0.05$ : Significantly different from the control.

should be considered together with the crop management, stock and processing, as these factors interfere in the components present in the mushrooms. Four *A. brasiliensis* varieties were tested, and the one with the best growth rate was selected for

this research (data not shown). In this study, the mushrooms were cultured by our associated farmer under an intensively controlled environment and medium. These media were tested to be free of heavy metals before being applied.

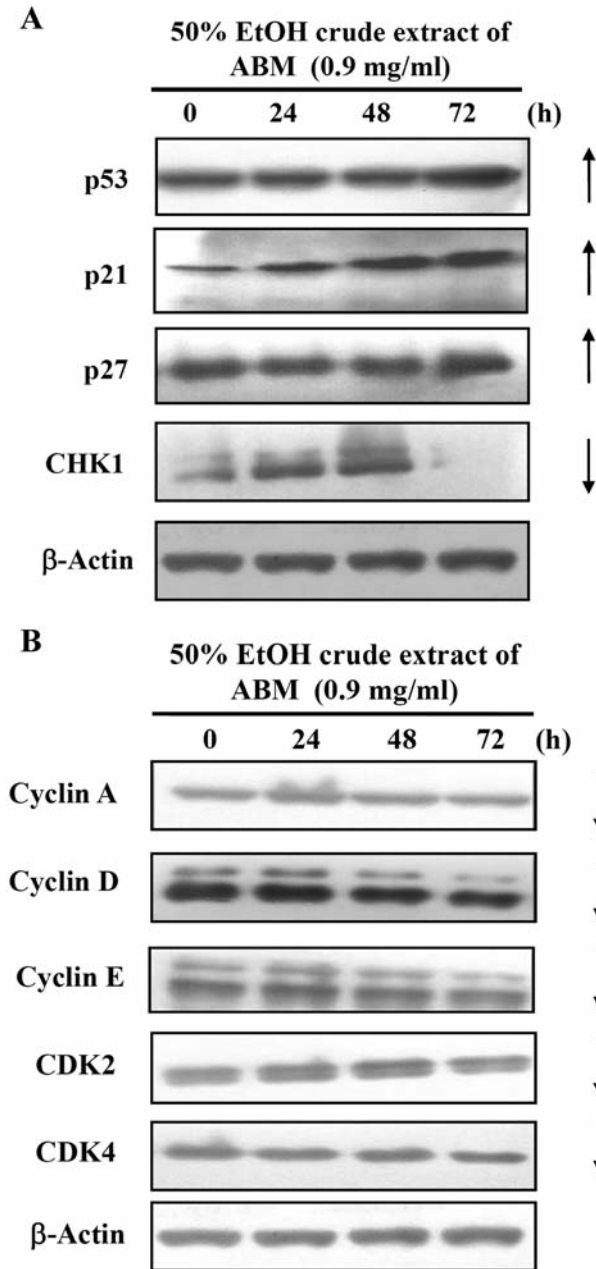


Figure 6. Effects of 50% EtOH crude extracts of ABM on G<sub>0</sub>/G<sub>1</sub> cell cycle arrest-associated proteins in CAL 27 cells by Western blotting analysis. Cells were treated with 0.9 mg/ml 50% EtOH crude extract of ABM for 0, 12, 24 and 48 h. The harvested and total protein were examined as described in the Materials and Methods for determining the protein levels of p53, p21, p27 and CHK1 (A) and cyclin A, cyclin D, cyclin E, CDK2 and CDK4 (B).

The effects of two different ABM extraction methods, 50% EtOH and hot water, on the growth of human oral cancer CAL 27 cells were also investigated. As a result, the hot water extract of ABM showed more potent inhibitive

activities compared with that of 50% EtOH extract, when evaluated by the cell viability (% of control by MTT assay) (Figure 1) as well as by the cell morphology (Figure 2). The inhibitory effect was greater at the concentration of 0.7 mg/ml for the hot water extract and at 0.9 mg/ml for the EtOH extract. The extraction methods used consequently do not appear to significantly affect the efficiency of ABM extracts on the apoptosis of human oral cancer CAL 27 cells.

Cell cycle distribution (Figure 3) and DNA fragmentation (Figure 4A) analyses showed that ABM extracts induced apoptotic cell death in human oral cancer CAL 27 cells. The G<sub>0</sub>/G<sub>1</sub> phase and sub-G<sub>1</sub> population increased, while that of the G<sub>2</sub>/M phase decreased after ABM treatment. Cells almost all stayed at G<sub>0</sub>/G<sub>1</sub> phase at 48 h after treatment with ABM extract (Figure 3). The gel electrophoresis of DNA showed a clear ladder at 48 h for cells treated with hot water, and at 72 h for treatment with EtOH extract (Figure 4A). All results indicated that ABM extract induces apoptosis in human oral cancer CAL 27 cells.

In order to further understand the molecular mechanism of ABM extract-induced apoptosis in CAL 27 cells, Western blotting assay was applied. The levels of p21, p27 and p53 proteins increased, while the levels of CHK1, cyclin D, cyclin E, CDK2 and CDK4 decreased. Such effects cause the cells to stay in the G<sub>0</sub>/G<sub>1</sub> phase. Mitochondria play an important role in cell apoptosis (43, 49). It was also found that the release of Ca<sup>2+</sup> in endoplasmic reticulum (ER) is related to the membrane potential of mitochondria (50). Ca<sup>2+</sup> is an important cell signal in apoptosis. At early stages of cell apoptosis, the Ca<sup>2+</sup> concentration increases (51, 52). When Ca<sup>2+</sup> is released from the ER, intracellular calcium homeostasis changes (53) and induces  $\Delta\Psi_m$  change and release of cytochrome *c*; increases of activity of downstream caspases then induces cell apoptosis (54-55). The level of Ca<sup>2+</sup> was increased when the CAL 27 cells were treated with ABM extracts (Figure 5C and D). This demonstrated that apoptosis was induced by ABM treatment through the mitochondria-dependent pathway. Assay of proteins related to apoptosis (Figure 6) indicated that ABM extract-induced cell apoptosis in CAL 27 cells may be caused by Ca<sup>2+</sup> release, decrease in  $\Delta\Psi_m$ , translocation of apoptosis-inducing factor (AIF) to the nucleus and induction of DNA fragmentation; or cytochrome *c* release from mitochondria activates caspase-3 and induces cell apoptosis, causing cells to die.

In conclusion, we demonstrated that ABM significantly induced apoptosis *via* reducing the levels of  $\Delta\Psi_m$ , induction of the release of cytochrome *c* and AIF from mitochondria, leading to the activation of caspase-3 and PARP degradation. The possible signal pathway involved in ABM extract effects are shown in Figure 8. Therefore, ABM might represent a promising agent for cancer chemoprevention and chemotherapy.  $\beta$ -Glucans from other



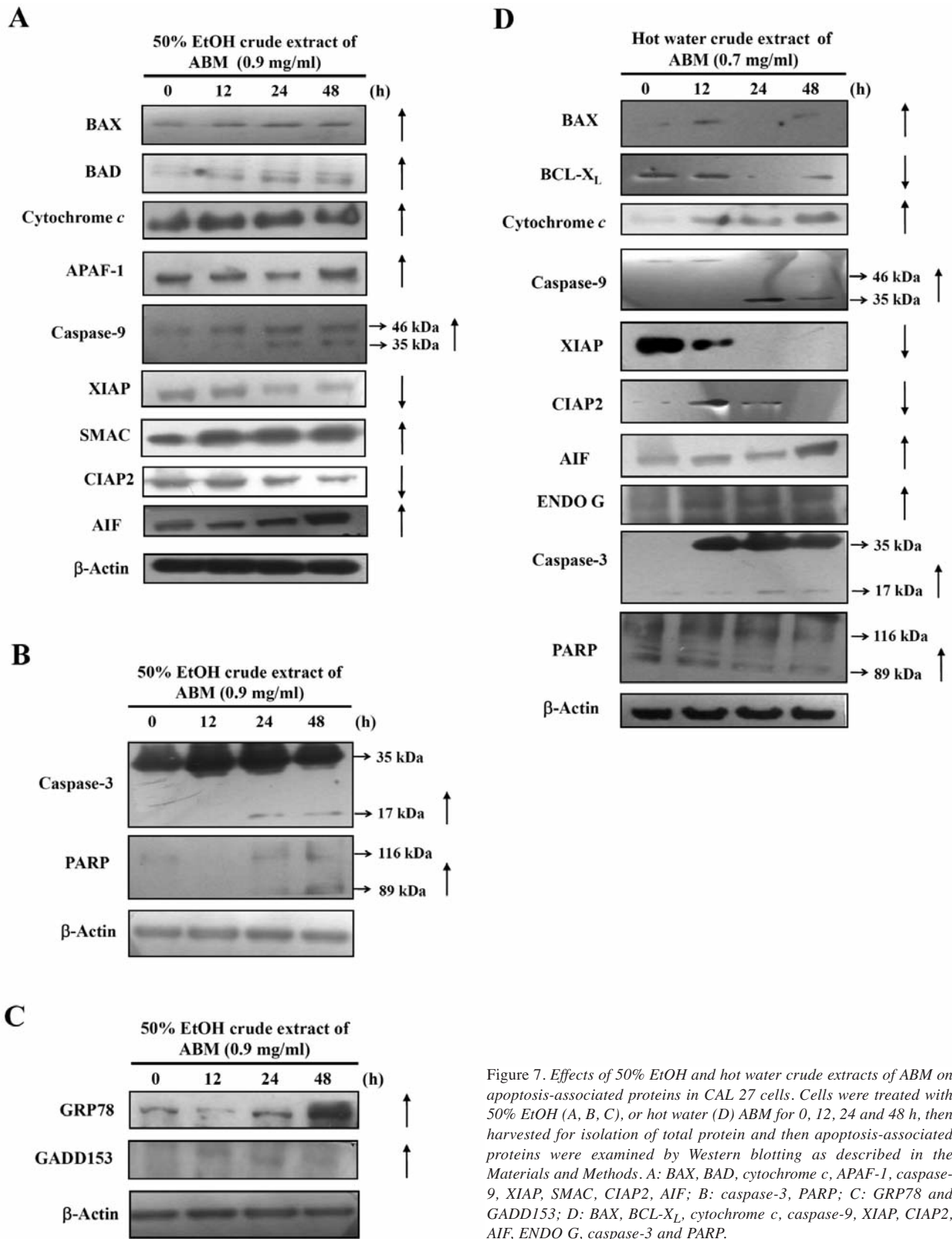


Figure 7. Effects of 50% EtOH and hot water crude extracts of ABM on apoptosis-associated proteins in CAL 27 cells. Cells were treated with 50% EtOH (A, B, C), or hot water (D) ABM for 0, 12, 24 and 48 h, then harvested for isolation of total protein and then apoptosis-associated proteins were examined by Western blotting as described in the Materials and Methods. A: BAX, BAD, cytochrome *c*, APAF-1, caspase-9, XIAP, SMAC, CIAP2, AIF; B: caspase-3, PARP; C: GRP78 and GADD153; D: BAX, BCL- $X_L$ , cytochrome *c*, caspase-9, XIAP, CIAP2, AIF, ENDO G, caspase-3 and PARP.

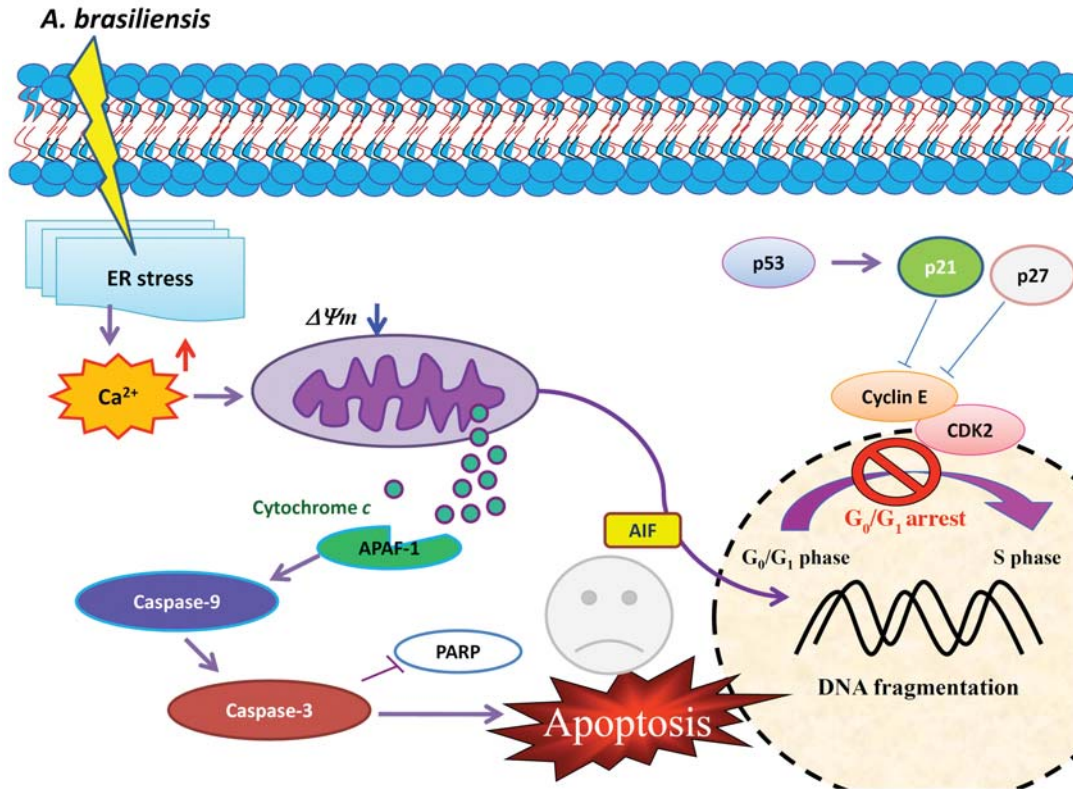


Figure 8. The signaling pathway of ABM-induced apoptosis appears to occur via reduction of  $\Delta\Psi_m$  levels, release of cytochrome c and AIF, caspase-3 activation, and PARP degradation in human oral cancer CAL 27 cells.

fungi have been shown to have antitumor, antidiabetic, antioxidant and anti-infection effects (16-19). The ABM extracts are rich in  $\beta$ -glucans that presumably contribute to the observed activity (9, 20), but other substances are probably involved as well.

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