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ANTRODIA CAMPHORATA GROWN ON GERMINATED BROWN RICE INHIBITS HUMAN HEPATIC CARCINOMA CELLS PPROLIFERATION VIA G0/G1 CELL CYCLE ARREST AND APOPTOSIS INDUCTION

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ABSTRACT

Antrodia camphorata (*A. camphorata*) is a parasitic fungus that has been used in traditional Chinese medicine because of its wide range of pharmacological activities. The purpose of this study is to investigate the antiproliferative effect and the mechanism of the ethyl acetate extract from *A. camphorata* grown on germinated brown rice (AGBR-EtoAc) in human hepatic carcinoma HepG2 cell lines. The results show that AGBR-EtoAc has significant remarkable inhibitory and antiproliferative effects on HepG2 cells in a concentration and time dependent manner. AGBR-EtoAc involved in the regulation of G0/G1 cell-cycle arrest and induced cell apoptosis. The protein levels of Cdk4 and cyclin D1 in the AGBR-EtoAc treated group were lower than those in the control group. Also, the expressions of the p53 and Bax were increased and Bcl-2 protein was downregulated with AGBR-EtoAc treatment. These findings suggest that AGBR-EtoAc extracts might act as an effective anti-proliferative agent by inducing G0/G1 cell cycle arrest and involved cell apoptosis in hepatic carcinoma cells.

KEYWORDS: *Antrodia camphorate, antiproliration, Cdk4, Cyclin D1, apoptosis*.

1.0 INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common lethal malignancies in the liver cancer, and is also one of the third most common causes of cancer death worldwide.[1] Various etiological factors have been found to be high-risked in association with HCC, including exposure to aflatoxin B1, and infection with hepatitis B and hepatitis C virus.[15,25] HCC treatment result is far from pleasing and overall survival rate of 5 years for approximately 10% of

people.[1] Although surgical resection is considered as the best treatment for liver transplantation, however; few patients with liver cancer have a surgically resectable disease and unrespectable HCC which are not suitable for transplantation or surgical resection.[28,7] More than 75% of patients proved to be potential candidates for chemotherapy. [4] Currently, chemotherapy using cytotoxic drugs, such as doxorubicin, cisplatin and fluorouracil is one of the most common treatments of choices, especially for patients with unrespectable tumors. However, because of low response rates, high recurrence rates and severe toxicities, the mean survival time is only approximately 6 months.[28,17] Therefore, it has been suggested that small-molecule targeted chemotherapy is a promising strategy to combat this cancer. Thus, there is a need to develop compounds that can effectively treat cancer.

A large number of medicinal mushrooms have been proven to possess anticancer activities.[10,13,16,30] One such mushroom, *Antrodia camphorata* has been used in conventional Chinese medicine. Traditionally, it has been used as a remedy for food-, alcohol-, drug-intoxication, diarrhea, abdominal pain, hypertension, skin itching, and liver cancer. In Taiwan, people recently used to improve liver function and to treat liver diseases.[2,11] Chang et al., reported the anti-proliferation effects of *A. camphorata* non-polysaccharide based extracts on human hepatoma cells.[5] Liquid state fermentation and solid state cultures of *A. camphorata* mycelia are now produced commercially for use as nutriceuticals.[27,26,18] Rao et al., suggested that chloroform extracts of *A. camphorate* can inhibit tumor growth in various types of cancer cell lines, especially in HepG2.[23]

Despite the clinical and pharmacological importance of *A. camphorata*, naturally occurring *A. camphorata* is not easily available in large quantities due to its high cost of production. Therefore, we developed novel methods for culturing *A. camphorata*. We successfully cultivated it on germinated brown rice (AGBR), which contains a large number of nutrients and active biological compounds. However, the activity of AGBR against liver cancer carcinogenesis has not been clearly well-understood. In this study, we investigated the effect of AGBR-EtoAc on HepG2 liver cancer cells and determined the molecular mechanism underlying this effect.

2.0 MATERIAL AND METHODS

2.1 Reagents and chemicals

Antrodia camphorata grown on germinated brown rice (AGBR); AGBR-hexane, AGBR-BuOH and AGBR-EtoAc extracts. RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA). Propidium iodide (PI), NP-40, and RNase A were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Anti-Cdk4, (Cell Signaling Technology Inc., Danvers, MA), anti-cyclin D1 (Cell Signaling Technology Inc., Danvers, MA), anti-Bcl2 (Cell Signaling Technology Inc., Danvers, MA), anti-Bax and anti-p53 (Cell Signaling Technology Inc., Danvers, MA), anti-β-actin, horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were obtained from the respective suppliers. The chemiluminescence detection kits (Amersham, USA) and MTT assay (Sigma, St. Louis, MO, USA) were purchased accordingly.

2.2 Preparation of AGBR

AGBR was grown as previously described. An authenticated voucher specimen of AGBR was deposited in the Herbarium at the Laboratory of Molecular Biomedicine, Institute of Bioscience, University Putra Malaysia (Serdang, Malaysia). Cultured mycelium of the *A. camphorata* was inoculated on germinated brown rice, and cultured at 20-25℃ for 4 weeks. The powdered material (1 kg) was extracted under reflux with 80% MeOH (methanol extract of AGBR for

48 h. The total extract (170 g, yield [w/w], 17.0%) was dissolved with water. After removing the insoluble solid particles by filtration, the liquid phase was extracted sequentially by solvents with increasing polarity (hex, EtOAc, BuOH, and water; 1:10 [w/v] for all solvents) to yield 4 fractions. The liquid-liquid phase extraction was performed in erlenmeyer flasks by shaking and the extracts were concentrated to dryness by a rotary evaporator. Thus, we obtained the following fractions: hexane fraction (15 g, yield (w/w) 1.5%), EtOAc fraction (4 g, yield (w/w) 0.45%), BuOH fraction (7.25 g, yield (w/w) 0.825%), and water fraction (11.86 g, yield (w/w) 1.086%).

2.3 Cell culture

The HepG2 cells (human liver cancer cells) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated FBS (Gibco, Grand Island, NY, U.S.A.), 100 U/ml of penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified incubator with 5% CO_2.

2.4 Cell proliferation assay

The effect of AGBR hexane, BuOH and EtoAc extract on HepG2 cell proliferation was measured using the MTT assay. The assay was performed as per the manufacturer's protocol. HepG2 liver cancer cells (1×10^4 cells/well) were placed in a 96-well plate and incubated with various concentrations (0, 50, 100 and 200 µg/ml) of AGBR hexane, BuOH and EtoAc extracts for 48 h and 72 h respectively. A fixed amount (10 µl) of MTT solution (5 mg/ml) was added to each well and incubated for an additional 2-4 h at 37˚C. Cell proliferation levels were detected at an optical density (OD) of 570 nm by using an ELISA Multi-Detection Reader (Tecan, Mannedorf, Switzerland).

2.5 DAPI staining

40, 6-Diamidine-20-phenylindole dihydrochloride (DAPI) staining was conducted according to the method described by Lock and others. Briefly, cells treated with AGBR-EtoAc for 48 h were harvested and washed twice with cold PBS, fixed with 4% ethanol for 30 min at room temperature, and then washed twice again with PBS. They were then incubated with 2 µg/ml DAPI solution for 30 min, and cell morphology was evaluated by fluorescence inverted microscopy. Cells with chromatin condensation, nuclear fragmentation, and nuclear condensation were recognized as apoptotic.

2.6 Flow cytometric cell cycle analysis

Cell cycle progression was determined using flow cytometery analysis. HepG2 cells (1×10^6 cells/ml) were incubated in 6-well plates in the presence or absence of AGBR-EtoAc extract for 48 h. Then, they were harvested by trypsinization, washed twice with phosphate buffered saline (PBS), and fixed with 70% ice-cold ethanol. After centrifugation, the fixed cells were incubated with a staining solution containing 0.2% NP-40, RNase A (30 µg/ml), and PI (50 µg/ml) (Sigma, St. Louis, USA) in a phosphate-citrate buffer (pH 7.2). Cellular DNA content was analyzed by flow cytometry using a Becton Dickinson laser-based flow cytometer (Becton Dickinson, New Jersey, USA). At least 10,000 cells were used for each analysis, and the results were displayed as histograms. The average percentage of cells in each phase of the cell cycle was determined over 3 independent experiments.

2.7 Western blotting analysis

The HepG2 cells were treated with AGBR-EtoAc extracts at various concentrations (0, 50, 100 and 200 µg/ml) for 48 h. 25 µg aliquots of protein were subjected to electrophoresis on 12% SDS-PAGE polyacrylamide gels and electrophoretically transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, Berkeley, California).

The membranes were incubated in 5% skim milk solution and then with antibodies against Cdk4, cyclin D1, Bcl2, Bax, p53, (1:2,000), and β-actin (1:5,000) for 2–3 h at room temperature with constant shaking. The membranes were washed thrice in a 1× PBS-T buffer and incubated with HRP-conjugated secondary antibodies (1:5,000) for 1–2 h. The membranes were washed and detection of the immunoreactive bands was performed using the enhanced chemiluminescence western blotting detection system (Amersham, USA).

2.8 Statistical analysis

Values are presented as percentage ± S.D. of control. Student's *t*-test or One-way ANOVA/Dunnett's *t*-test was used to analyze the statistical significance between the AGBR-EtoAc treated and control groups. Statistical analysis was performed using SPSS, version 12 (SPSS Inc., Chicago, IL, USA).

3.0 RESULTS

3.1 Anti-proliferative activity of AGBR in HepG2 hepatoma cell lines

In comparison with the various extracts of AGBR-Hexane, AGBR-BuOH and AGBR-EtoAC showed a significant inhibitory effect on the growth of HepG2 cells (fig1 a). After 48 h of treatment with 200 μg/ml of AGBR-hexane, AGBR-BuOH and AGBR-EtoAc extracts, the HepG2 cell proliferation was reduced by 79.67% ± 2.27, 65.46% ± 2.46 and 46.35% ± 4.85, respectively. Therefore, further experiments were conducted using the AGBR-EtoAc extract. As shown in (fig1 b), AGBR-EtoAc extract significantly inhibited HepG2 cell proliferation in a concentration and time dependent manner. However, AGBR-EtoAc extract did not affect cell viability of mouse macrophage RAW 264.7 cell (data not shown).

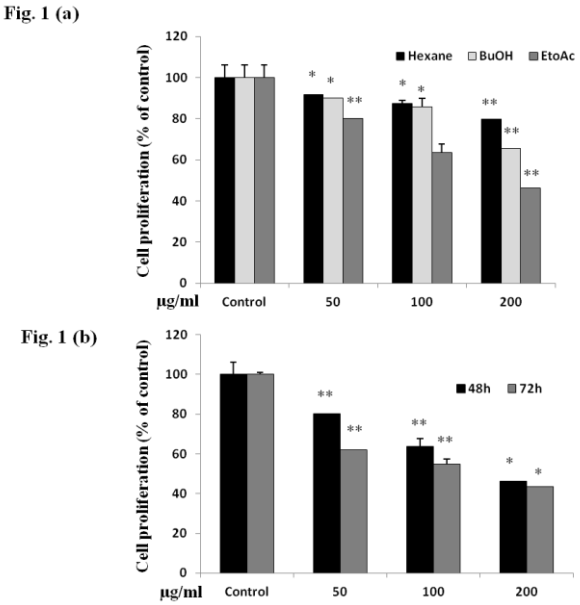

Figure 1: Anti-proliferative effect of AGBR (hexane, BuOH and EtoAc) extracts on HepG2 cells. (a) HepG2 cells (1 x 10^4/well) were treated with various concentrations (0, 50, 100, and 200 µg/ml) of AGBR various extracts for 48 h. The HepG2 cell proliferation was determined by the MTT assay. One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's t-test ($^{*}p< 0.05$; $^{**}p< 0.01$ vs. control). (b) Effect of AGBR-EtoAc extracts on HepG2 cell proliferation. Time and dose dependent inhibition of HepG2 cell proliferation by the AGBR-EtoAc extracts. The growth inhibition was calculated as a percentage of inhibition compared with the control. One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's t-test ($^{**}p< 0.01$ vs. control). AGBR, Antrodia camphorata grown on germinated brown rice.

3.2 DAPI staining

We focused on the morphological change of apoptotic nuclei induced by AGR-EtoAc using DAPI staining (fig 2). Uniform HepG2 cells with normal morphology were observed in control group, whereas HepG2 cells exhibited condensed and fragmented nuclei, which is indicative of apoptosis, were noted following treatment with AGBR-EtoAc (0, 50, 100 and 200 µg/ml) for 48h. These results suggest that AGBR-EtoAc is capable of inducing marked apoptotic morphological changes in HepG2 human hepatoma cells.

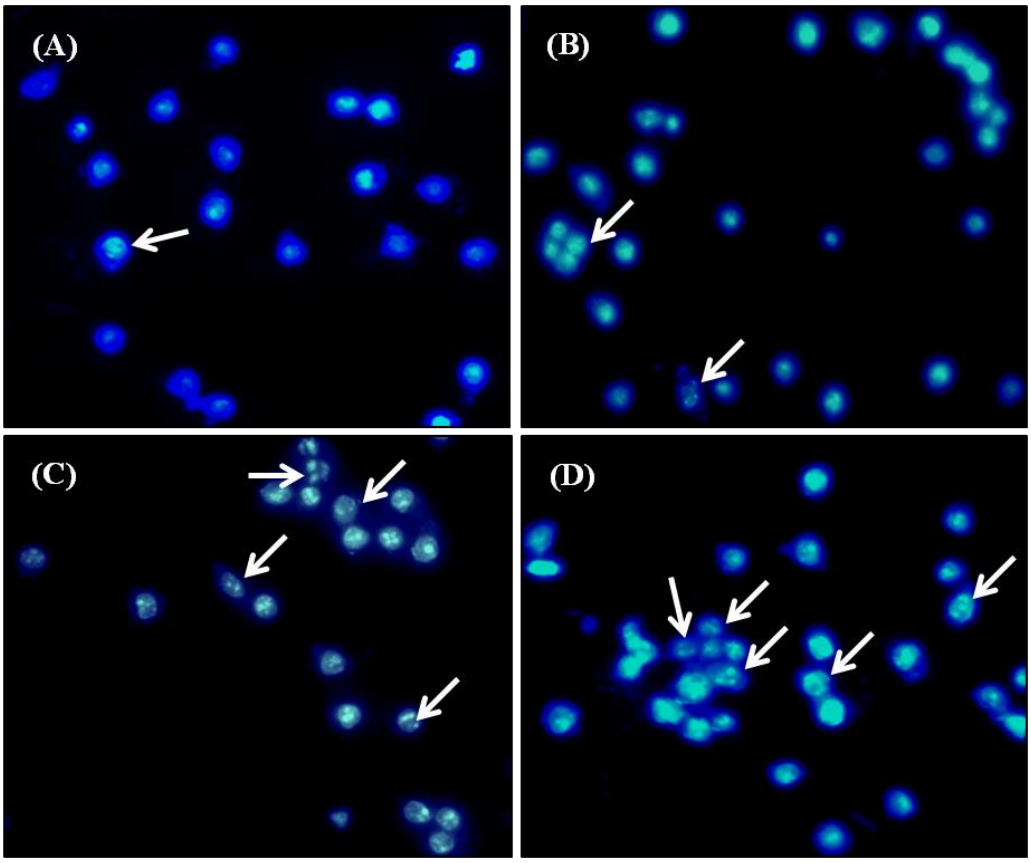

Figure 2: The effect of AGBR-EtoAc on morphological changes in nuclear (x100). (A) Control cells without AGBR-EtoAc treatment; (B) Cells treated with 50 µg/ml (C) Cells treated with 100 µg/ml and (D) Cells treated with 200 µg/ml of AGBR-EtoAc treatment for 48h, then the differentiation criteria of nuclei were studied by DAPI staining.

3.3 Cell cycle and apoptosis analysis by flow cytometry

To assess the effect of AGBR-EtoAc treatment on the distribution of cells, we performed cell cycle analysis. As shown in fig 3, apoptotic cells (Sub-G1) significantly increase in a concentration dependent manner. Compared to the control (50.41%), AGBR-EtoAc treatment resulted in a significant increased of cells in the G0/G1 phase of the cell cycle by 53.80%, 63.71% and 68.75% when treated with 50, 100 and 200 µg/ml concentrations of AGBR-EtoAc extract, respectively. These results suggest that AGBR-EtoAc induces apoptosis via cell cycle arrest in G0/G1 phase.

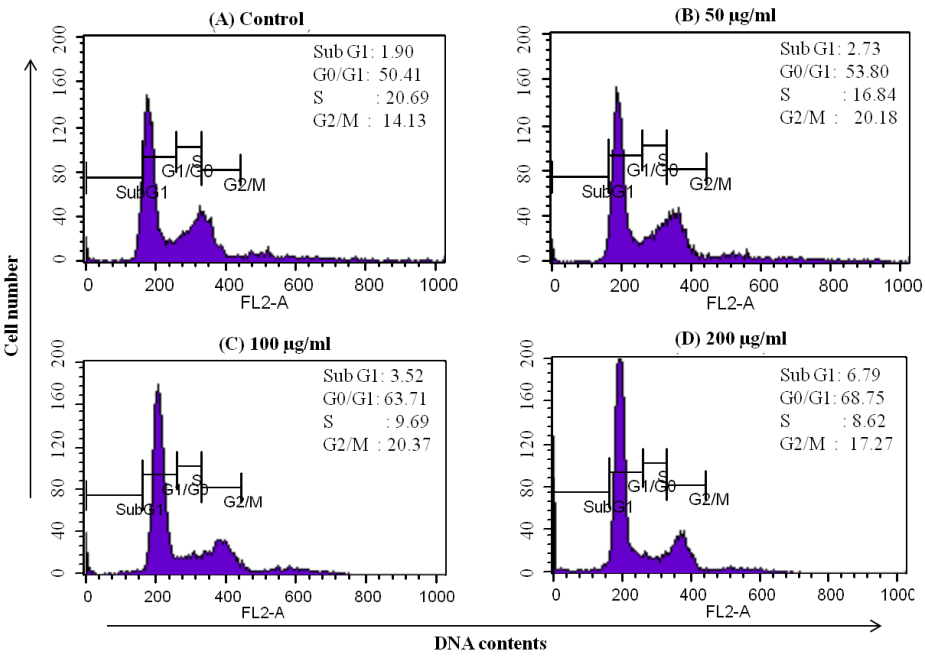

Figure 3: Effect of AGBR-EtoAc on cell cycle progression of human HepG2 liver cancer cells. Cells were treated with various concentration of AGBR-EtoAc extract for 48 h. Cellular DNA was stained with PI solution. Flow cytometric analysis was performed to analyze the cell cycle distribution. Data shown are representative at least three independent experiment.

3.4 Effects of AGBR-EtoAc extract on the expression of cell cycle regulatory proteins

In order to investigate the mechanisms of AGBR-EtoAc involved in the regulation of G0/G1 cell-cycle arrest related proteins, we assessed G0/G1check point proteins in AGBR-EtoAc extract treated cell cycle arrest for 48 h. As shown in fig 4, the expression levels of Cdk4 and cyclin D1 that are involved in the G0/G1 cell-cycle progression were markedly down-regulated in cells treated with 50-200 µg/ml of AGBR- EtoAc. Therefore, the results indicate that the cell-cycle arrests in G0/G1 phase.

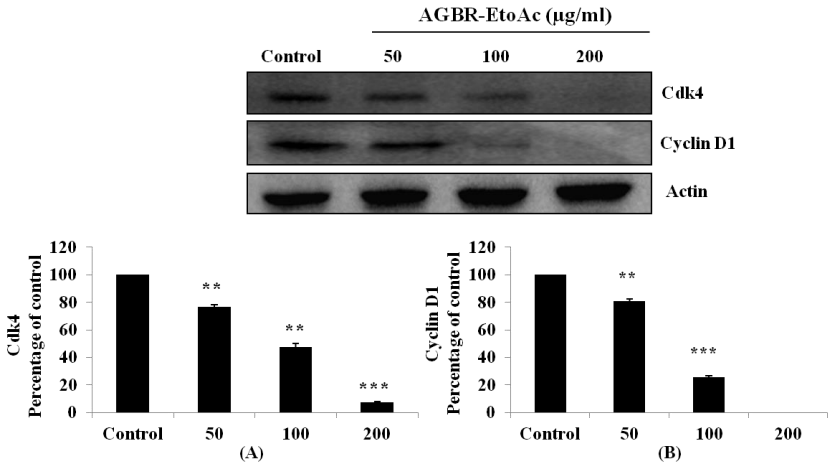

Figure 4: The effects of AGBR-EAC on the expression of G0/G1 phase cell-cycle regulatory proteins in HepG2 hepatoma cell lines. Cells were treated with the indicated concentrations (control, 50, 100 and 200 µg/ml) of AGBR-EAC for 48 h. Cell lysates were processed for western blot analysis with anti-Cdk4, anti-cyclin D1 and β-actin antibodies. Densitometric (A and B) analysis of three independent western blots (mean ± SD) expressed in terms of a percentage of the values for the control groups ($^{**}p< 0.01$; $^{***}p< 0.005$).

3.5 Changes of p53, Bax, and Bcl-2 Proteins in HepG2 Cells

To determine whether tumor suppression factors such as p53 and Bax; and antiapoptotic protein such as Bcl-2 were involved in antiproliferative effect of AGBR-EtoAc extract on HepG2 cells, the levels of protein were analyzed by western blot (fig 5). Expressions of p53 and Bax protein significantly increased in the cells with AGBR-EtoAc treatment in a concentration-dependent manner. In contrast, Bcl-2 protein expression was remarkably downregulated with AGBR- EtoAc treatment.

Figure 5.

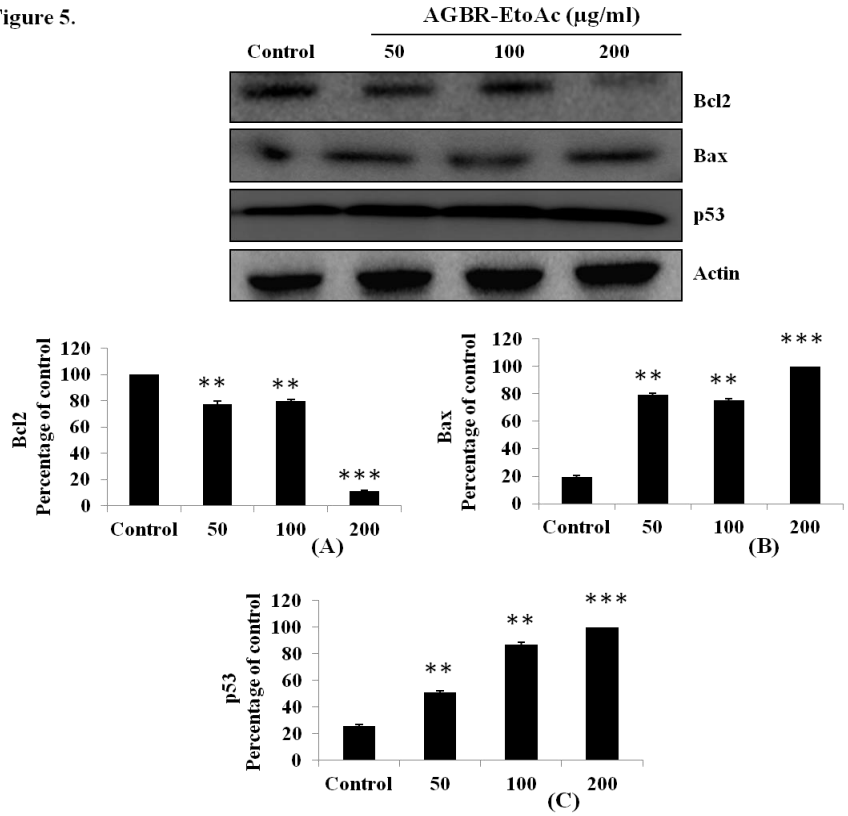

Figure 5: The effects of AGBR-EAC on the expression of apoptosis-related proteins in HepG2 hepatoma cell lines. Cells were treated with the indicated concentrations (control, 50, 100 and 200 µg/ml) of AGBR-EAC for 48 h. Cell lysates were processed for western blot analysis with anti-p53, anti-Bcl2, Bax and β-actin antibodies. β-actin was used as an internal control. Densitometric (A-C) analysis of three independent western blots (mean ± SD) expressed in terms of a percentage of the values for the control groups ($^{**}p< 0.01$; $^{***}p< 0.005$).

4.0 DISCUSSION

Various studies have indicated that the induction of apoptosis might be due to cell cycle arrest.[29] Therefore, inhibition of the cell cycle has been appreciated as a target for the management of cancer.[20,21,9] In the present study, we demonstrated that AGBR- EtoAc plays a key role in several of physiological and pharmacological functions of cell proliferation.[22] The proliferation inhibitory effects of AGBR-EtoAc against human liver carcinoma cell lines and the possible mechanism. To the best of our knowledge, this study is the first to investigate proliferation by inducing G0/G1 cell cycle arrest and apoptosis via the mitochondrial pathway.

Inhibition of tumor growth has been a continuous effort in cancer treatment. Reduction in cell growth and the induction of cell death are two major means to inhibit tumor growth.[12] This study demonstrates that AGBR-EtoAc extracts has

anti-proliferatives against HepG2 cells. Although AGBR (hexane and BuOH) extracts exhibite anti-proliferatives against HepG2 cells, AGBR-EAC extracts showe anti-proliferative with the lowest IC_{50} (200 μg/ml) value for 48h. It has also been found that the viability of HepG2 cells are inhibited by treatment of AGBR-EtoAc extract.

Apoptosis and necrosis are involved in cell death. There are some key features for apoptosis, which are common to different cell death pathways. Apoptosis includes cell shrinkage and loss of contact with neighboring cells, formation of cytoplasmic vacuoles, membrane blebbing, chromatin condensation, DNA fragmentation, and formation of apoptotic bodies.[3] Microscopic examination of stained cell nucleus is the most direct and reliable test, since it makes it possible to perform high-quality studies of cell morphology, nuclear and chromatin disintegration, as well as to distinguish viable or apoptotic and necrotic cells.[24] In the present study, DAPI staining results show morphological features such as cell condensed and fragmented nuclei, which are clearly evident after 48h treatment of AGBR-EtoAc compared to that of control cells.

Disturbance of the cancer cell cycle is one of therapeutic targets for development of new anticancer drugs.[6] From our study, we found that activation of AGBR-EtoAc reduces the proliferation of cells through the arrest of cell in G0/G1 phase. It has been demonstrated that the proliferation of cell decreased gradually following the increase of AGBR-EtoAc concentration in culture. Cyclin D1 and Cdk4 has most important roles in blocking the cell cycle in the G1 phase.[14] Protein level of cyclin D1 and Cdk4 decreased following treatment of liver cancer cells with AGBR-EtoAc, supporting their contribution as a possible mechanism by which these agents exhibit inhibitory growth.

The upregulation of p53 and Bax played a key role in the induction of apoptosis in cancer cells. Normal p53 plays an important role in inducing apoptosis and cell cycle checkpoints in human and murine cells following DNA damage.[19] This has further been supported by the finding that p53 is the most common tumor suppressor gene. The p53 protein plays a crucial role in cell cycle control and induction of apoptosis during the treatment period in cancer patients.[8] Bcl-2 is an upstream effector molecule in the apoptotic pathway, the proapoptotic and antiapoptotic members of the Bcl-2 family act as a regulating programmed cell death. Therefore, the ratio of Bax/Bcl-2 is a final factor that plays an important role to determine whether cells will undergo apoptosis. In the present study, AGBR-EtoAc downregulates Bcl-2 protein expression while at the same time upregulates Bax protein. In addition, the identification of such compounds will improve our understanding of the antiproliferative activities of AGBR-EtoAc. Further experiments need to be done to clarify the anti-proliferative mechanisms of these identified compounds.

5.0 CONCLUSION

The finding of the present study indicates that AGBR-EtoAc can efficiently suppress the growth of human liver cancer cells in association with cell-cycle arrest in the G0/G1 phase. The treatment of HepG2 cells with AGBR-EtoAc results in significant: cell growth inhibition, G0/G1-phase cell-cycle arrest by regulating cell-cycle-related factors, apoptosis through the mitochondrial pathway and modulation of Bcl-2 family protein. These findings suggest that AGBR-EtoAc may be applicable for the medical treatment of hepatocellular carcinoma (liver cancer). Further study is needed to investigate the effect of AGBR-EtoAc on hepatocellular carcinoma (liver cancer) *in vivo*.

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