

Full Length Research Paper

Enhancement of induced apoptosis in human melanoma A375 by a combination of natural compounds

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Accepted 17 May, 2011

Jaceosidin, emodin and magnolol are natural compounds isolated from Chinese herbs and are reported to have anticancer properties; although a combination of natural anticancer drugs could increase the efficacy of anticancer activity. However, the combination of these compounds was examined by using MTT assay, cell cycle arrest, flow cytometry and western blot on melanoma A375. The results showed that the new combinations induced cell cycle arrest in G2 phase and apoptosis through Bcl-2 expression. Emodin enhanced the anticancer activity of jaceosidin and magnolol; as such, a combination of emodin with magnolol was more effective. These observations indicated that a combination of emodin with magnolol and jaceosidin enhanced anticancer activity through induced apoptosis by Bcl-2 expression, while a combination of emodin with magnolol was more effective than other combinations and could be considered further in future researches of melanoma.

Key words: Emodin, jaceosidin, magnolol, apoptosis, melanoma.

INTRODUCTION

Although melanoma incident is on the increase worldwide, the chemotherapy treatments still cannot increase the prevention of melanoma metastasis and have caused harmful side effects to the human body (Crosby et al., 2009). Furthermore, the natural compounds are promising chemotherapy agents used to treat melanoma in the future and they require depth researches and investigations to discover and develop new, single or combination chemotherapy agents that can raise the response ratio of melanoma and cancer treatment (Amit et al., 2001; Azadeh et al., 2010). However, screening of traditional Chinese medicine produced natural compounds that have anticancer properties, although some compounds have been used under clinical trials (Xiaojuan et al., 2005). Also, screening of TCMs exhibited

an anticancer activity against melanoma, in that TCMs have been proven to induce apoptosis in melanoma *in vitro* (Chyi et al., 2007; Xu et al., 2009). Combination of natural compounds that have anticancer properties is used to exhibit new agents, followed by increasing efficacy of response ratio of chemotherapy treatment, but the future trends of chemotherapy are still needed in order to focus on the decrease of side effects which is the main problem of chemotherapy treatment (Natalie and Rebecca, 2006; Kyaw et al., 2010).

Jaceosidin is a natural compound isolated from *Artemisia argyi* and approved to have antioxidant, anti-inflammatory and antiallergic properties (Min et al., 2008). Also, it was reported to have anticancer properties (Lv et al., 2008; Jeong et al., 2007; Lee et al., 2005). Magnolol is a natural compound isolated from the root and stem bark of *Magnolia officinal* and which exhibited antifungal, antibacterial and antioxidant properties (Lo et al., 1994; Kwon, 1997; Bang et al., 2000). Also, it was reported to have anticancer properties (You et al., 2009; Ikeda et al.,

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2003). Emodin is a natural compound isolated from *Rheum palmatum* and *Polygonum cuspidatum* and could reduce the impact of type 2 diabetes (Shun et al., 2005; Xue et al., 2010). Also, it was reported to have anticancer properties (Lai et al., 2009; Lin et al., 2009). A combination of these three natural compounds (jaceosidin, magnolol and emodin) has no report against tumors and melanoma. From the previous studies, new combinations of jaceosidin, emodin and magnolol were examined to know if they enhance the anticancer properties against melanoma A375 and if they were approved by cell cycle arrest, flow cytometry and western blot.

MATERIALS AND METHODS

Cell culture

Melanoma cell line (A375) was purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in culture in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 100 unit of Antibiotic–Penicillin at 37°C in a humidified incubator in 5% CO₂–95% air (CO₂ incubator).

Drugs preparation

Jaceosidin was isolated and identified by using HPLC, mass spectrum, H1-NMR and C13-NMR techniques from Chinese herb (*Folium Artemisia Argyi*) via the screening method from the library of Chinese herbs on melanoma A375 for the first time. Emodin and magnolol were purchased from Shanghai AoBo Bio-Pharmaceutical Technology Co., Ltd (China). The drugs were dissolved in DMSO to a concentration of 20 mM and then to sub-concentrations of 10 to 50 µM.

AO/EB staining to detect apoptosis

Staining of A375 was done by Acridine orange/ethidium bromide (AO/EB) staining (AO and EB were purchased from Bio Basic, INC, China) to detect apoptosis, and the cells were harvested in 1 x 10⁵ to 2 x 10⁵ cells per ml of 96 wells/cells plate, after 24 h of treatment with combination of jaceosidin (50 µM), emodin (10 µM) and magnolol (50 µM). We started the staining procedure by removing the medium and adding 8 µl of AO/EB (1:1) (The dye mix for the EB/AO staining was 100 µg/ml acridine orange and 100 µg/ml ethidium bromide in PBS) for each well, after which the medium was gently mixed and incubated for 10 to 20 min at 37°C. Then, it was observed and photos were taken by using optical microscopy (Deborah et al., 2005).

Cell proliferation assay

Cells were cultured in a 96-well-plate for the first 24 h. They were incubated with a combination of jaceosidin (50 µM), emodin (10 µM) and magnolol (50 µM). After 24 h of drugs treatment, the medium was removed and 10 µl of MTT was added to each well. After incubation at 37°C for 4 h, the medium was removed and 100 µl of DMSO was added to each well to dissolve purple crystals of formazan. The plate was shaken for 10 min to allow complete solubilization. Spectrophotometric absorbance at 570 nm was read

on a 96-well plate reader and we followed the equation below to determine the values of inhibition growth:

$$\% = [A570 (\text{control}) - A570 (\text{treated})] / A570 (\text{control}) \times 100$$

Cell cycle arrest

Cells were treated with a combination of jaceosidin (50 µM), emodin (10 µM) and magnolol (50 µM) into a 6 well-plate. Twenty-four hours later, the cells were collected, washed and re-suspended in PBS, after which they were centrifuged (5000 rpm for 3 min) and the PBS was removed and treated with cold 70% ethanol. They were then froze immediately at -20 for 24 h. Twenty-four hours later, the ethanol was removed and the pellets were washed by cold PBS twice, before RNase and PI (purchased from Sangong Company, China) were then added to the samples and analysis by using Becton Dickinson FACS Calibur flow cytometer via Cell Quest software.

Cell apoptosis assay

Cells were treated with a combination of jaceosidin (50 µM), emodin (10 µM) and magnolol (50 µM) in a 6 well-plate. Twenty-four hours later, the cells were collected, washed and re-suspended in PBS. Apoptotic cell death was identified by double survival staining with recombinant FITC (fluorescent isothiocyanate)-conjugated Annexin V and PI, using the Annexin V-FITC Apoptosis Detection kit (purchased from Beyotime Biotechnology, Hainen, Jiangsu, China). According to the manufacturer's instructions, flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed in a Becton Dickinson FACS Calibur flow cytometer using Cell Quest software.

Western blot analysis

Cells were treated with different concentrations (0, 30 and 50 µM) of jaceosidin, magnolol and emodin combinations into a 6 well-plate for 24 h. Twenty-four hours later, cells were washed twice with PBS and lysed in lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF dissolved in isopropyl), after which loading buffer was added and then centrifugation was done at 12,000 rpm for 10 min. The total protein content of each supernatant was determined with a Bio-Rad protein assay kit. An equal amount of protein of each sample was added to sodium dodecyl sulfate sample buffer and proteins were separated by polyacrylamide gel (12%) electrophoresis. Following electro transfer to PVDF membranes, the membranes were blocked overnight with 5% non-fat dry milk and 0.1% Tween20 in 0.01M Tris-buffered saline (TBS).

After three washes with TBS containing 0.1% Tween20 (T-TBS), membranes were incubated for 2 h at room temperature Bcl-2 (diluted 1:300) (purchased from Biosynthesis Biotechnology CO.LTD, Beijing, China) and β-actin antibodies (diluted 1:500) containing T-TBS. After several washes with T-TBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (purchased from Sigma, USA) at a dilution of 1:500 for 1 h at room temperature. After washing with T-TBS, proteins were visualized using an enhanced chemiluminescence detection system (purchased from Millipore Corporation, Billeica, USA). Thus, X-ray films were used to detect the proteins expression.

Statistical analysis

Data were expressed as means ± SD, and analyzed by the Student's t-test on SPSS 17.0. P-values below 0.05 were

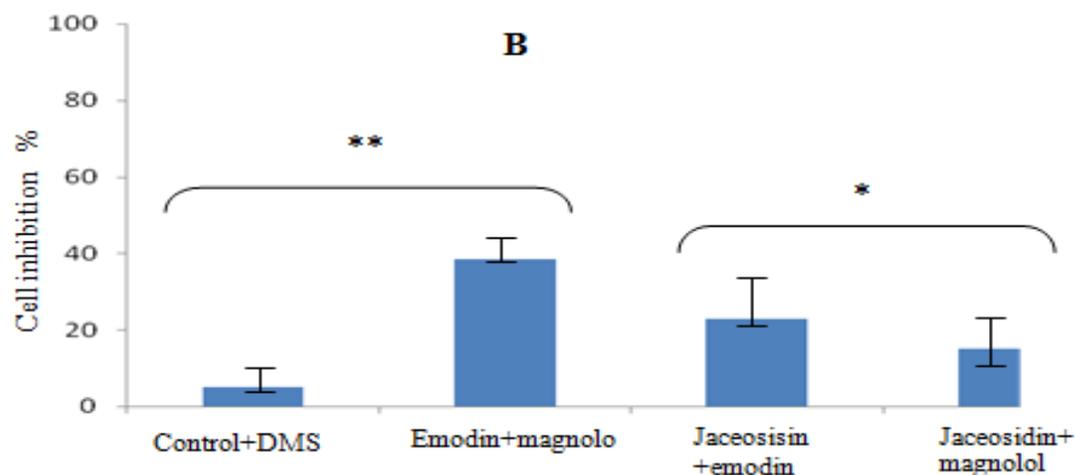
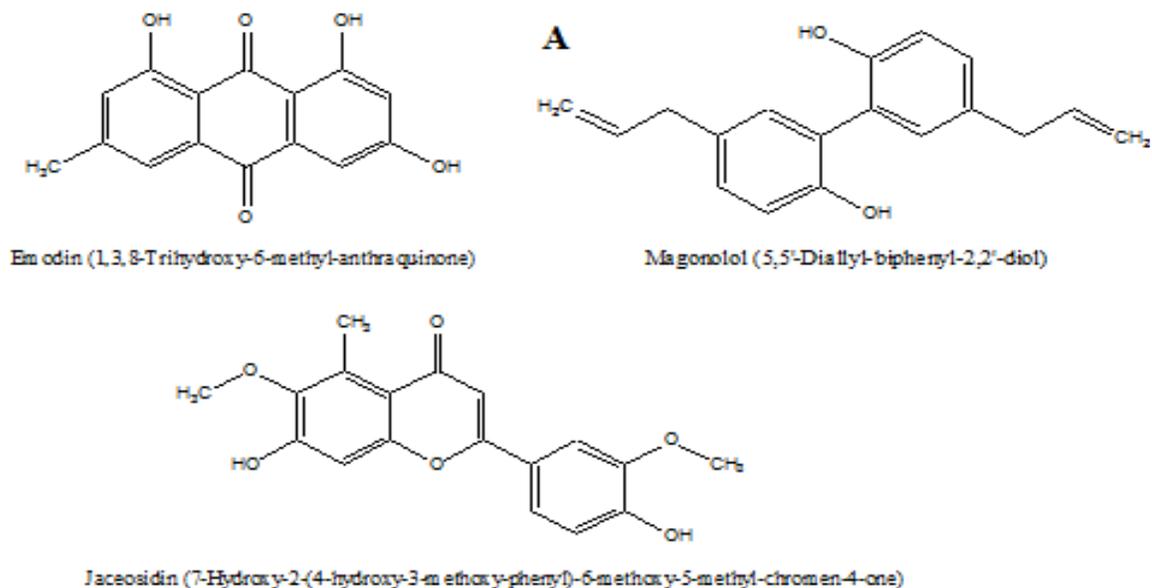


Figure 1. (A) Chemical structures of emodin, magnolol and jaceosidin. (B) Growth inhibition of jaceosidin (50 μ M), emodin (10 μ M) and magnolol (50 μ M) combinations after treatment for 24 h. Data represent mean \pm SD of triplicates (* P < 0.05; ** P < 0.01).

regarded as statistically significant.

RESULTS

Effect of combinations on proliferation inhibition of melanoma A375 cells

Cells were treated with a combination of jaceosidin (50 μ M), emodin (10 μ M) and magnolol (50 μ M) for 24 h. Twenty-four hours later, we added MTT assay and measured it by absorbance reader at 570 nm. The results showed that combination of emodin with magnolol is higher than other combinations (emodin with jaceosidin;

and jaceosidin with magnolol), as shown in Figure 1B.

Effect of combinations on cell cycle arrest of melanoma A375

Cells were treated with a combination of jaceosidin (50 μ M), emodin (10 μ M) and magnolol (50 μ M). Twenty-four hours later, cells were analyzed by using flow cytometry to detect G0/G1, S and G2/M ratios, and the results showed that G2/M phase increased in the drugs' combinations with a reduction of G1 and S when compared with the control. However, a combination of emodin with magnolol was more effective than other

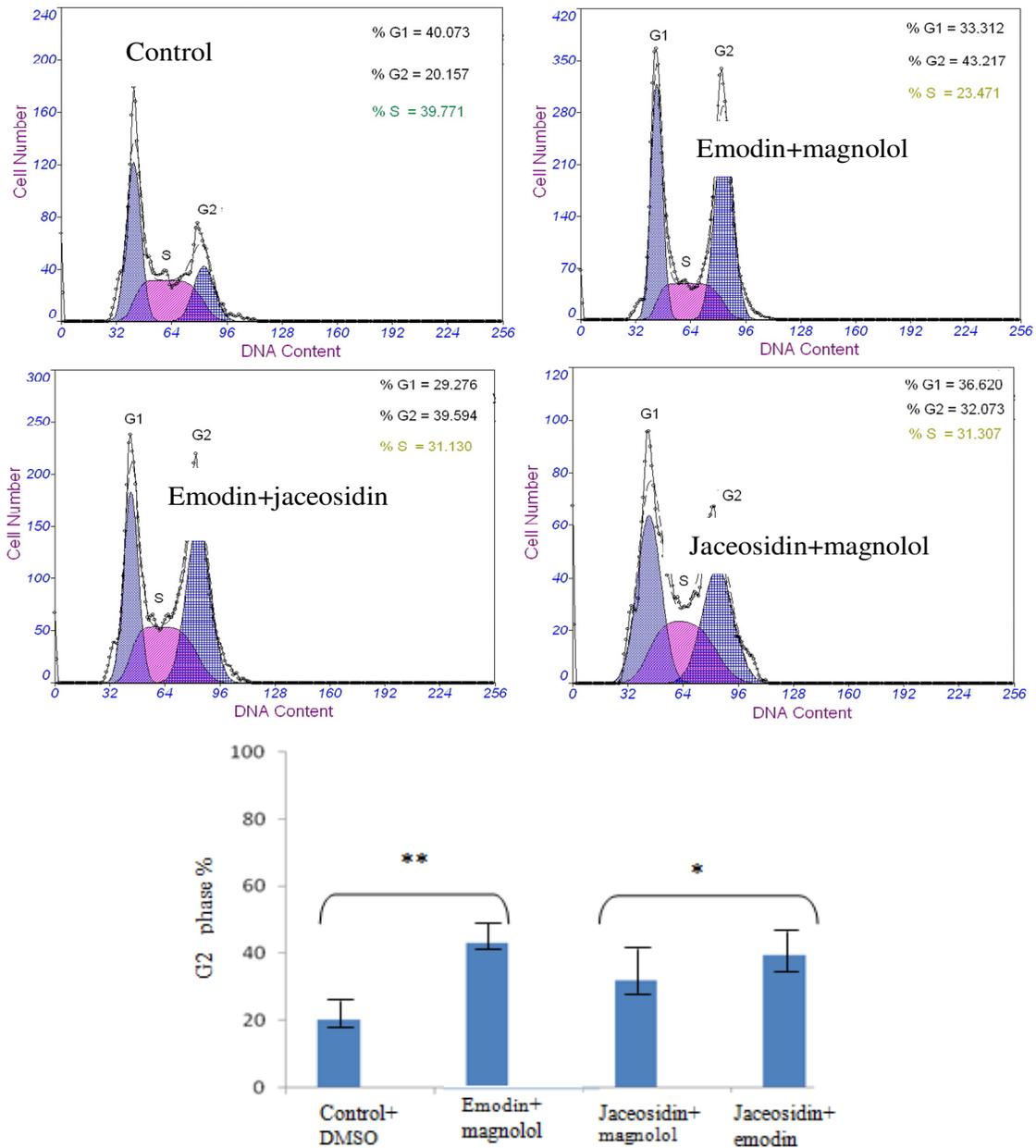


Figure 2. Cell cycle arrest after 24 h of jaceosidin (50 μ M), emodin (10 μ M) and magnolol (50 μ M) combination treatment. Data represent mean \pm SD of triplicates (* P < 0.05; ** P < 0.01).

combinations, as shown in Figure 2.

Apoptosis detection by AO/EB staining

Cells were grown in a 96 well plate and were treated with combinations of jaceosidin (50 μ M), emodin (10 μ M) and magnolol (50 μ M) for 24 h. The plate was stained by AO/EB stain (1:1) and the effect was observed by using optical microscope, which showed live (green cells), necrosis (red cells) and early and late apoptosis (bright green followed DNA fragmentation) cells. Also, it showed

that a combination of emodin with magnolol and jaceosidin was more effective on apoptosis than jaceosidin with magnolol, as shown in Figure 3A.

Effect of combinations on apoptosis of melanoma A375

Cells were treated with a combination of jaceosidin (50 μ M), emodin (10 μ M) and magnolol (50 μ M) for 24 h. Twenty-four hours later, cells were analyzed by using flow cytometry to determine the live, necrosis, early and

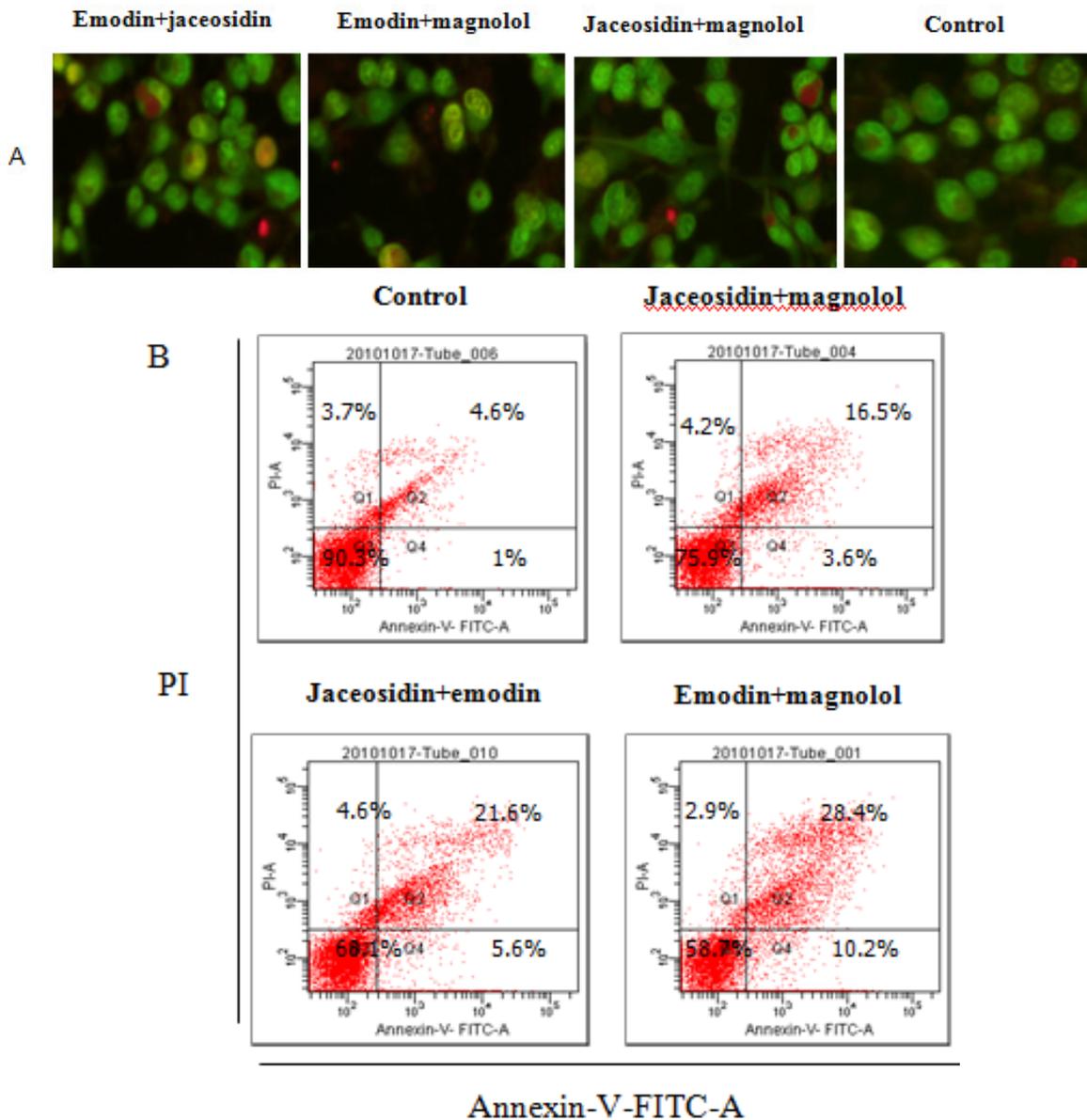


Figure 3. Treatment of cells with a combination of jaceosidin (50 μ M), emodin (10 μ M) and magnolol (50 μ M), and incubation for 24 h. (A) Treatment with AO/EB stain to detect live (green), necrosis (red) and apoptosis (bright green and DNA fragmentation) cells. (B) Affected apoptosis by a combination of jaceosidin, emodin and magnolol. Data represent mean \pm SD of triplicates (* $P < 0.05$; ** $P < 0.01$).

late apoptosis ratios. However, it was observed that emodin with magnolol combination was higher than other combinations, as shown in Figure 3B.

Effect of combinations on expression of Bcl-2 in melanoma A375

To investigate whether drugs combination induced apoptosis through Bcl-2 pathway or not, we used 0, 30 and 50 μ M of drugs combination for 24 h, and then extracted the protein, after which immunoblotting was

used to detect the expression of Bcl-2, as shown in Figure 4.

DISCUSSION

A combination of three natural compounds belonging to three different families (flavones, quinines and phenols) having different chemical structures (different function groups can play a role in increasing the anticancer properties) was examined on melanoma A375, as shown in Figure 1A. The results of proliferation showed that a

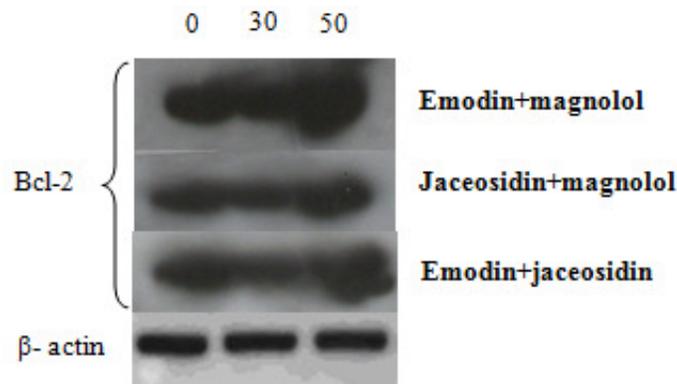


Figure 4. Cells were treated with different concentrations (0, 30, and 50 μM) of jaceosidin, magnolol and emodin combinations for 24 h to detect induced apoptosis by Bcl-2 expression.

combination of emodin with magnolol has high growth inhibition (38.53 ± 1.16) than other combinations (jaceosidin + emodin = 23.00 ± 0.45 ; jaceosidin + magnolol = 15.16 ± 0.45) when compared with the control (5.16 ± 0.76), as shown in Figure 1B. This result indicates that a combination of emodin with magnolol and jaceosidin could enhance the efficacy of drugs ability to inhibit cells viability in melanoma A375. In cell cycle arrest, results showed that the G1 phase (emodin + magnolol = 33.30 ± 0.42 ; jaceosidin + magnolol = 36.81 ± 0.29 and jaceosidin + emodin = 29.23 ± 0.65) was reduced when compared with the G1 phase control (40.73 ± 0.74), although slight changes were observed for the S phase. Also, the results showed that G2/M phase (emodin+magnolol = 43.22 ± 1.51 ; jaceosidin + magnolol = 32.073 ± 0.63 and jaceosidin + emodin = 39.59 ± 0.46) increased when compared with G2/M phase control (20.157 ± 1.46). These values indicated that the three combinations were held by the cell cycle at G2/M phase. However, a combination of emodin with magnolol exhibited high G2/M than other combinations, as shown in Figure 2.

Furthermore, we used AO/EB staining to detect apoptosis affected by drugs combination, and the observation which was done through a optical microscope showed live (green cells), necrosis (red cells) and early and late apoptosis (bright green followed by DNA fragmentation) cells (Leite et al., 1999). The staining showed that a combination of emodin with jaceosidin and magnolol was more effective than jaceosidin and magnolol, in that it clearly exhibited DNA fragmentation, as shown in Figure 3A. By using Annexin-V-FITC and PI staining, we detected early and late apoptosis ratios and the results showed that the combination of emodin and magnolol induced the early and late apoptosis ($38.6 \pm 0.90\%$) higher than the early and late apoptosis of other combinations (jaceosidin + emodin = $27.2 \pm 0.70\%$, jaceosidin+magnolol = $20.1 \pm 0.88\%$) when compared with the control ($5.6 \pm 0.52\%$). These results indicate that a

combination of emodin with magnolol and jaceosidin could enhance induced apoptosis of A375 cells, while a combination of emodin with magnolol is more effective than other combinations on apoptosis, as shown in Figure 3B.

Also, to know whether the combination of drugs could induce apoptosis, we used immunoblotting to detect the expression of Bcl-2 protein by using 0, 30 and 50 μM of drugs combination. It was observed that a combination of emodin with magnolol and jaceosidin at 50 μM concentration enhanced the expression of Bcl-2 more than jaceosidin and magnolol combination, while at 30 μM concentration, slight expression of Bcl-2 was shown. Thus, we compared the expression with β -actin as a control. The results suggested that emodin combined with magnolol and jaceosidin enhanced the induced apoptosis by Bcl-2 expression as shown in Figure 4.

Conclusion

Our experience in screening the library of Chinese traditional medicine as anti-melanoma created a belief that TCMS could find new, single or combination agents to treat melanoma in the near future. The results of the study suggested that emodin combined with jaceosidin and magnolol enhanced anticancer activity through induced apoptosis by Bcl-2 expression more than magnolol and jaceosidin combination. Also, a combination of emodin and magnolol is more effective than other combinations against melanoma A375. However, the findings of these combinations may provide further use and studies of melanoma researches.

ACKNOWLEDGEMENT

The authors are grateful to Northeast Normal University, Membrane Channel Research Laboratory for providing the materials and molecular techniques and for technical supports.

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